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REVIEW

EVOLVING SCENARIO OF PHARMACEUTICAL CARE IN PAKISTAN AND OTHER COUNTRIES: HEALTH IMPACT ASSESSMENT IN PUBLIC HEALTH PRACTICE

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Abstract: Worldwide, pharmaceutical care has been recognized as the primary mission of pharmacy. According to the philosophy of pharmaceutical care, pharmacist is not only responsible to dispense the medicines but also responsible to improve the patient's quality of life. Pharmaceutical care practice is required to be introduced in the developing countries to decrease drug related mortality and morbidity. This paper aimed to highlight the quality of pharmaceutical care practice in the developing countries, predominantly in Pakistan. The paper highlights the health status and current scenario and barriers to pharmaceutical care practice in Pakistan. Pharmacists in Pakistan are not much involved in the provision of pharmaceutical care services due to a number of barriers that include insufficient number of pharmacists, lack of proper time, inadequate skills and training, lack of financial support and limited recognition of pharmacists in the public. A majority of community pharmacies are running without a pharmacist under the supervision of unprofessional personnel.

Keywords: pharmacist, pharmaceutical care, medicines, quality of life, barriers, skills

Hepler and Strand provided first concept of pharmaceutical care (PC). Pharmaceutical care is the responsible provision of drug therapy to achieve definite outcomes that improves the patient's quality of life (1). Pharmaceutical care is an important component of pharmacy practice which is directly concerned to the patient care (2).

Around the world, PC has been promoted as a standard for patient care provision in the last few decades (3). Pharmacy organizations and academic training programs have played an important role in promoting this concept (3). Because of PC, the concept of pharmacy profession has been transformed more specifically to patient care that was aimed to achieve the positive outcomes for the patient from drug therapy (4). In past few years, the concept of pharmacy profession was limited to compounding and dispensing of medicines. As time passed, the compounding role of pharmacist was decreased and pharmaceutical care emerged as a new role of pharmacy profession (5). Pharmaceutical care is aimed to assure the safe and effective use of medicines by patient's drug therapy monitoring and to organize health awareness programs for the society (6, 7). Whenever drugs are given, the potential for suboptimal outcomes which diminish the patient's quality of life is always present. These suboptimal outcomes might be resulted due to a number of reasons but inappropriate monitoring is the most important cause (8).

In PC practice, the basic component is mutual-beneficial conversation between the patient and PC provider (pharmacist responsible for PC services). The patient trusts on the PC provider as a reliable source of drug information giving him authority to improve his therapeutic outcomes. In this way, pharmacist accepts his responsibility by rendering his commitment and competence (9). The PC provider is never meant to replace the dispensing pharmacist, the physician, the nurse or any other health care professionals. Pharmaceutical care provider is a new health care provider for the patient (10). PC can help in improving the patient's therapeutic outcome that includes improved drug therapy and disease management, greater patient safety, reduced health care expenditure, better patients' adherence and compliance and improved quality of life. This concept has also been related to the issues of safety of patient explored by World Health Organization (WHO) (11). It is not only sufficient to properly prescribe as non-adherence may

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occur due to insufficient drug information, incomplete labeling, financial problem and cultural perception related to drugs (12). Complete and proper delivery of drug information is equally important as that of prescribing and pharmacist is responsible for it (13). In disease management patient's education is considered to be the most important tool in spite of scientific and technological progress. Patient needs proper education regarding the drug dose, frequency and route of administration. Patients should be advised regarding the unwanted effects of medicines. Pharmaceutical care provider should ensure that the patient received the medications adequate to their clinical needs, in doses that meet their requirements, for an appropriate duration of time and at the economic cost to them and their community (12).

Pharmaceutical care - the primary mission of pharmacy profession

Internationally, PC has been recognized as the primary mission of pharmacy. The concept of PC needs be applied in all settings such as hospital, community, clinic and long term care. It is useful for the patients with all types of diseases and getting any type of therapy (1).

Pharmaceutical care in disease management

Health does not mean the absence of a disease only, but also mean the social, physical and mental fitness. Therapeutic outcomes of drug therapy directly affect psychological, social and physical health domains that, in turn, influence the health related quality of life. Adherence to medication and modification in diet and life style has significant effects on therapeutic outcomes, which is only possible by implementing PC practice (14).

The Portuguese pharmacist association (ANF) has introduced certain tools and methods for disease management programs which includes: software applications, documentation forms, and pharmacist's intervention protocols. These disease management programs are mainly focusing on patient counseling. Nowadays, PC programs are being organized for asthmatic, diabetic and hypertensive patients. After an extensive research efforts, a number of new developments have been conducted and some of the results were published in 2004 and 2005 (15).

Pharmacists are comfortable in providing disease oriented PC to patients than the comprehensive PC. However, it is under-discussion that whether PC should be provided to a certain group of patients with specific diseases or to all others (16, 17).

Pharmaceutical care has been found to be useful in chronic disease management including hypertension, diabetes, asthma and human immunodeficiency virus (HIV).

Pharmaceutical care in hypertension

In UK, the results of a pilot study revealed that PC implementation has effects on control of blood pressure and rational prescribing of statins and antiplatelet agents for coronary heart failure prevention and arthrosclerosis prevention (18). Another study conducted in Poland, explored the positive impacts of PC on patient's knowledge about hypertension (19). Hypertension management involves medication adherence, long-term management plans, and drug-related problem control. Patient's adherence is affected by the side effects of drugs that ultimately affect the therapeutic outcome (20). PC program has been found to be effective in improving the adherence and medication compliance in hypertensive patients (21). In UAE, a randomized trial explored the significant positive impact of pharmaceutical care program on the clinical and humanistic outcomes in heart failure patients (22).

Pharmaceutical care in diabetes

Several studies have been conducted to evaluate the impact of PC program on diabetes type I and type II patients. Diabetes management includes optimal glucose control that can be made possible by strict adherence to drug therapy, exercise and diet. Pharmaceutical care is most effective in the field of diabetes as some of studies have explored the poor knowledge of diabetes patients regarding their disease (diabetes), long term complications and its management (20). In Switzerland, a PC model has been developed for type II diabetes by Wermeille et al. (23). In Turkey, a short-course pharmaceutical care program resulted in improved blood glucose level and systolic and diastolic blood pressure control (24).

Pharmaceutical care in asthma

A number of studies have been conducted to evaluate the influence of PC on asthmatic patients in several countries, including Germany, Finland, Denmark, Malta, Netherland and Spain and showed successful and positive effects on economic and health care outcomes (25). A twelve month controlled intervention study resulted in significant improvement in inhalation technique, quality of life, self-efficacy and patients' disease specific knowledge (26). Another randomized controlled study reported the positive impacts of pharmaceutical care on asthma patients (27).

Pharmaceutical care in HIV

Pharmaceutical care has been found to be effective in management of health conditions of HIV patients. In HIV patients, several drug related problems (DRPs) have been identified and resolved in some studies (28, 29). In Nigeria, PC interventions resulted in significant improvement in HIV patient's immunological and virological outcomes which highlighted the role of pharmacists in therapy management and patient's care (30).

Pharmaceutical care in cancer

All over the world, cancer is considered to be one of the major health problems. Cancer diagnosis is difficult and patient needs special care and considerations. In Saudi Arabia, a cross-sectional study was conducted to evaluate the impact of pharmaceutical care practice on quality of life of cancer patients. Patients' health related quality of life was found to be improved which showed that improved cancer patient's care and quality of life can be achieved by improvement in clinical oncology pharmacy services (31).

Pharmaceutical care in hospital pharmacy setting

Pharmacists' provided hospital pharmacy services must include pharmaceutical care as an efficient component to assure the improved patient's health care outcomes and quality of life.

Major responsibility of hospital pharmacist is to ensure medication use process that includes prescribing, compounding, dispensing, administration and monitoring of medications for patient's safety and therapy effectiveness. Around the world, several ways and technologies were adopted to deliver these services.(32, 33).

Since 1990, clinical pharmacy became an important practice in Italy. Pharmaceutical care appeared as dominant practice in hospital pharmacy settings but very little literature is available about the community pharmacy practice development in Italy (34). It is difficult to get an overview of pharmacy practice in Switzerland due to the presence of federal structure and different languages spoken in the country. In 1996, some activities were started for improvement of pharmacy practice and creation of new opportunities in PC but it seemed that only limited implementation was made due to poor research efforts (35). Pharmaceutical care practice is required to decrease drug related mortality and morbidity in developing countries. World Health Organization has approved certain guidelines that were adopted by the International Pharmaceutical Federation for successful implementation of good pharmacy practice in developing countries (36).

The situation of pharmacy practice in developing countries varies from one country to another, even the situation varies between different regions within a country (37). In comparison to developed countries, the profession of pharmacy is still not well known in developing countries because the pharmacists have never been given their place in health care settings neither by the health care professionals nor by the community (38).

In Nigeria, the concept of clinical pharmacy was introduced in 1980 when some hospital pharmacists were directed to provide clinical services such as unit dose dispensing and drug information service. In Nigeria, still most of pharmacists are not willing to provide PC services, although PC has become an ideal approach of pharmacy practice. After about two decades, pharmacy practice in community and hospital pharmacies has suffered from poor infrastructure, poor staffing, and deficiency in proper coordination in activities. Pharmacists having poor confidence and training, conflict with physicians, and failure of pharmacist to accept the PC practice (39). Nigerian pharmacists are now accepting the practice of PC, while the importance of its application is repeatedly discussed in ongoing workshops, conferences and education programs.

In Mexican health care system, pharmacists have not been recognized and accepted as a member of health care team. There is still lack of drug monitoring, insufficient drug information centers and limited collaboration of pharmacists and other health professionals in hospitals (40).

In Saudi Arabia, a survey study was conducted at regional level in hospital pharmacies of Riyadh. The results showed an increased use of electronic technologies by hospital pharmacists to improve prescribing and transcribing services (41). Hospital pharmacists were found actively involved in medicines therapy monitoring and patient's education in Riyadh (42). In UAE, pharmacists work in various health care settings which include hospital pharmacy, community pharmacy, and drug information and drug distribution. To meet high demand for pharmacists, many of them are hired from other countries. In public sector, a few clinical pharmacists are working while private sector is deprived of them. Therefore, PC is at initial stages of development and requires more attention to meet the needs of patients (43). In China, clinical pharmacy practice has been started in 2005 to improve the PC services and hospital pharmacy settings. In some hospitals of China, clinical pharmacists have worked to standardize daily work routines and developed the standard operation chart for training of clinical pharmacy stu-

dents and medication orders (44). A three months interventional study was conducted in China by placing a pharmacist in medical ward. The study has reported the positive effects of PC on patients' outcomes and reduced medication errors (45).

Pharmaceutical care in community pharmacy setting

Internationally, community pharmacies are involved in providing a range of PC services to their consumers, particularly in developed countries. As a result of interest of National Health Service (NHS) in new possible roles of pharmacist, a large number of studies were conducted to assess the opinion and needs of patients and pharmacists. In current scenario of health practices, consultant pharmacists are performing medication review in addition to community and hospital pharmacists (46). Practitioners like pharmacists and nurses were given training for time-being supplementary prescription writing (46, 47).

In 1993, PC program was introduced in Sweden (15), but the main focus of Apoteket (National Pharmacy Organization) was on counseling, over-the-counter (OTC) drugs and health promotion advice. For drug related problems, a national database was found which accelerated the process of drug review implementation. Pharmaceutical care was further supported by the availability of a national register of patients' dispensed drugs in the later years (16).

Since 1995, PC has become a part of professional standards for community pharmacy practice in Denmark (48). Though, the privacy issues related to drug-data has hindered its application just like in Sweden. Nowadays, only a few pharmacies are working in an organized way for the detection of drug related problems (49), in spite of long research activities and implementation of PC (particularly in the field of migraine and asthma) (50). In Netherland, patients expect that community pharmacists will provide them with pharmaceutical care services. Here the patients usually visit the same pharmacy to get a good quality medication counseling and automated medication surveillance service (51).

In Belgium, registered drug dispensing requires a doctor's prescription and pharmacists are not allowed to substitute the branded drug by a generic drug (52). For community pharmacist, PC provision has become a legal duty but due to the shortage of pharmacist, implementation of PC remained difficult (53). Limited information is available regarding health developments in France due to inaccessibility of most of French journals on internet database and sometimes in libraries. In France, Dupin-Spriet and Wierre in 2004 found the possibilities for medication management review. For motivation of pharmacist to implement more drug surveillance and to apply PC in their practices, the French Order des Pharmaciens is doing its best (54).

In Spain's legislation, the responsibility of PC provision has been given to community pharmacist. A study conducted by Sigma Dos reported the highest level of satisfaction with the pharmaceutical care services provided in community pharmacies (55). It resulted in classification of drug-related problems and a system of drug review (56). Limited research in pharmacy practice has been found in Spain, in spite of a large number of operational community pharmacies. In 2005, some advanced cognitive services were found but few of them were paid (57).

In developing countries, a very little literature is available regarding the standards of pharmacy practice in community pharmacy. Community pharmacy is readily and easily approached by the general public so the PC services should be provided in community pharmacy (58).

In Nigeria, several studies have been carried out regarding the PC practice. A study explored the positive attitude of Nigerian pharmacists towards PC practice. In Benin City, it was reported that some of the community pharmacists were involved in practicing some PC activities such as blood pressure monitoring and medication history taking (59).

In Peru, patient counseling is an uncommon practice until patients asks. Some of pharmacy chains are now involved in providing drug information to patients through e-mails and phone calls. A few pharmacies are taking part in public health campaigns through provision of information about socially relevant products including correct use of condoms and emergency contraception (60).

In Brazil, National Agency of Sanitary Vigilance (ANVISA) is responsible to monitor the good pharmaceutical practice (GPP) and regulate pharmaceutical care in community pharmacies (61). Most of the Brazilian pharmacies are involved in providing drug dispensing services including patient counseling. Pharmacist can substitute a generic drug for the prescribed medication. Like in other countries, most of pharmacies are working in absence of a pharmacist; therefore, the quality of dispensing is of substandard. Limited data is available regarding the impact of PC on humanistic, economic or clinical outcomes in community pharmacies (62).

In Uganda, components of community pharmacy practice has not been quantified but anecdotal

evidence suggests that community pharmacy practice mainly involves drug dispensing services with limited pharmaceutical care services. Therefore, the customers visits to pharmacy and expectations to collect over the counter (OTC) medicines or to fill the prescription with less pharmaceutical care component (63).

In Turkey, community pharmacies are private enterprises and are legally required to be owned and supervised by pharmacists. One pharmacist is legally allowed to run/own only a single pharmacy where the pharmacists and pharmacy employees play their role as the dispensers. Most of the dispensers are untrained employees dispensing without the supervision of a pharmacist. It is legal requirement that pharmacy managers and owners should take registration from the regional board of pharmacists. Pharmacists presence is necessary during the opening hours of the pharmacy which is not strictly followed (12).

In Palestine, community pharmacies are easy to approach and are comparatively economical. Thus, most of the patients preferably seek health advice directly from the community pharmacies than the doctor's clinics, especially in case of urban areas where health services are underdeveloped. As a result, community pharmacies have to play an important role in community and public medical issues. Most of the community pharmacists are not up-to-date in pharmacology and clinical pharmacy issues; therefore, they are incapable of providing adequate medical advice and drug information on common medical problems (64).

The role of community pharmacists in PC provision is still indistinct in Iran. A number of pharmacies are restricted to the prescription filling only. Limited numbers of pharmacists are involved in patient education. Mostly patients are not informed about drug's storage conditions, adverse drug reactions, drug interactions and precautions (65). Many of pharmacists cannot identify the drug interactions and are unable to check the prescription's legality due to certain reasons including insufficient knowledge, deficiency of professional development programs, and more interest in trade than responsibility (66).

According to the philosophy of PC practice in China, pharmacist is responsible to provide the patient-centered services to improve the patients' quality of life (67). For primary healthcare promotion in China, understanding of prolonged role of pharmacists and community pharmacies is important. In last few decades, this country's pharmacy profession has faced considerable change. Nevertheless, only a limited data are available on current status of PC practice in China, where community pharmacies are acting as a primary health care source (68).

In India, the PC services are explored to a very little extent. For proper counseling of patient, lack of proper training of the pharmacists was found (69). In 2005, it was found that 50% of pharmacies were running without pharmacists (25), and a large number of patients (70-80%) get advice about contraceptive methods, sexually transmitted diseases, menstrual disorders and minor illnesses from community pharmacist. Like in other developing countries, an economical source of medical care in India is the private community pharmacy setting (70).

Health status of Pakistan's population

Among most populous countries of the world, Pakistan stands at 6th position with an estimated population of 184.35 million in 2012-2013 (71), and by the year 2050, population will be ranked as one of the largest (fifth position) populous country of the world with population of 285 million (72).

In Pakistan, the expectancy of life for women is 66 years and for men 64 years (73). In the list of newborn death rate, Pakistan stands at the top 8th position around the world, which means that one in each 10 children born in Pakistan dies before the age of 5 years while the chances of women's death during pregnancy and child birth is one in eighty (74). According to an estimation, the percentage of underweight children who are < 5 years of age is 38%, whereas the percentage of children who are severely underweight is 12% (75). An estimated number of 67000 people are living with HIV in Pakistan. The maternal mortality is difficult to measure due to its high rate which is much more in rural areas than that of urban areas that is 319 per 100,000 in rural areas and 175 per 100,000 in urban areas (74).

In Punjab and Khyber Pakhtunkhwa (KPK), the coverage of services has improved with the passage of time, but the gap between the health facilities of urban and rural areas remained as such in Balochistan and Sindh (74). As an example, in 2010/11, the full immunization coverage rates was about 77% in KPK, 79% in rural Punjab, but only 45% and 67% in remaining two provinces of Pakistan i.e., Balochistan and Sindh, respectively (74).

The health care system of any country is dependent upon the availability of human resources. Clear and long term vision is needed for the development of human resources in Pakistan. For this important health system, no responsible units are

found in federal Ministry of Health and the Provincial Departments of Health and no organized system for disease surveillance (73).

In Pakistan, the health care system is divided into two distinct sectors; private and public sector. The public sector provides health care *via* three ways; primary, secondary and tertiary. The primary level includes basic health units, rural health centers, primary health care centers, first aid posts, dispensaries, lady health workers and mother and child health centers. The secondary level consists of Tehsil and District Headquarter Hospitals while the teaching hospitals are involved in tertiary care provision (76). Provincial government has the responsibility to ensure the health except in federally administered region. Formulating and planning the national health policies is the responsibility of Federal government (76).

The main health care providers are doctors, nurses, pharmacists and assistant pharmacists, who are 116,298, 48,446, 8102 and 31,000 in number, respectively. The number of health care providers is less in proportion to the population. However, the physicians are dominant and hold major positions in administrative and decision making policies (77).

Due to lack of integration, lack of managerial delegation, political interference and deficiency of human resource management, the public health sector is underutilized. It is observed that 70-80% of Pakistani population, specifically those settled in tribal and rural areas, utilize alternative and complementary medicines due to their affordability, easy access, family demand and community suitability (78). Medical emergencies and undesirable health outcomes are observed due to inadequate and delayed health care (79). For the improvement of population's health status, there is a need to integrate the traditional and modern health care systems after appropriate training of traditional health sector.

Pharmaceutical care practice in Pakistan

The value of PC practice in community and hospital pharmacy of Pakistan has been explored very little as compared to other developing countries. It is difficult to induce those results to pharmacy settings in Pakistan due to the difference in health care system (80).

An estimated number of qualified pharmacists working in Pakistan is 8102, of which 5023 are practicing in private sector, 2836 in public setting and 243 in private but not-for-profit organizations (81). About 70% of pharmacists are working in pharmaceutical industry while just 10 percent are working in community pharmacies (82). The community pharmacies are estimated to be 63,000 but unfortunately, the number of pharmacists working in community pharmacies is fewer and cannot focus on PC, patient counseling and health promotion (81). These pharmacies are running with unsatisfactory conditions. The personnel working there have 10 to 12 years of schooling education with little or no professional training. This personnel with limited knowledge is performing the duties of an inventory manager, dispenser, prescriber, patient counselor and information provider. The principal challenge for the country is the current health scenario. For community pharmacy practicing, a large number of country's pharmacists rent out their category license for a monthly payment to the laymen. Currently, the non-professional persons run the community pharmacy/retail/medical store. These persons have insufficient knowledge about drugs (83). Moreover, the general public is unaware of the pharmacist's role in patient care (84). It is observed that the consumers describe their symptoms to this lay personnel who provides them treatment for their medical problem. The handling of prescription is poor and patients are treated even without a prescription. This all is due to shortage of pharmacists.

A qualitative study conducted by Kousar et al. (85) explored the pharmacists' attitude towards the PC practice in community pharmacies in Khyber Pakhtunkhwa, a province of Pakistan. The results of study showed the lack of community pharmacists' participation in direct patient care, patient counseling, and poor collaboration among the health care professionals. A study of Punjab province, revealed the availability of insufficient number of community pharmacists that resulted in inappropriate patient counseling and insufficient public awareness of pharmacist's role (71).

Pharmacy practice laws exist in Pakistan, but unfortunately are improperly implemented due to weak regulatory framework and lack of accountability (80). Here, national data on patient's self-medication is unavailable, but some studies disclosed that this malpractice varies between 6.3-51.3% depending on settings. Although with proper guidance, self-medication can be useful (86).

Going through this scenario, a question arises that either the pharmacist are willing to provide PC services in Pakistan or not (84).

Barriers to practice pharmaceutical care in Pakistan

Pakistan as well as other developing countries are facing certain barriers in PC implementation. The present review will highlight some of major

barriers faced by the pharmacists and pharmaceutical care practice. Insufficient number of pharmacists in both community and hospital pharmacies are reported in developing countries like Pakistan. A majority of the private and few public hospitals are running without hospital pharmacists. Pharmacists are limited to drug dispensing and procurement and are not involved in patient care activities (87).

One of the major barriers to practice and implement the PC practice is the lack of effective collaboration among the health care professionals. Patients' health condition can be improved by setting a good and effective collaboration among the health care providers (88).

Time is another barrier to practice and implement of PC services. Pharmacists do not have enough time to plan for PC. A mixed methods study by Murtaza et al. (87) explored the time constraint as a major cause of non-provision of patient counseling.

Documentation is an important parameter required for PC continuity, research and reimbursement (89). Due to limited technological/software resources, it is difficult to maintain patients' history and prescription record.

Hospital and community pharmacists are having inadequate skills to fulfill the needs of PC, which is due to limited resource of pharmacist's training. Lack of financial support and encouragement for provision of PC services is another important barrier (90). Other barriers include people's wrong perception regarding pharmacist and poor knowledge of patients about drugs due to their low level of education (91).

Another issue is the uneven distribution of health personnel within and between countries. High proportion of health personnel are reported in wealthier and urban areas. Great disparities in health outcomes are resulted due to uneven distribution of health personnel between urban and rural population. Health care providers are more attracted to urban areas for their comparative professional, social and cultural advantages. Big cities offer more chances for educational and career development, easier access to private sector and lifestyle relevant facilities and services. Economics is the most important factor which affects the decision of professionals whether to leave or stay. Job-related decision can be affected by a number of factors including personal, educational, professional and social matters (92). In underserved and rural areas, health professionals are not ready to accept positions due to lack of facilities, equipment, and health supplies. Medical students also avoid to practice in rural areas due to these issues (93). Another consideration is the mix of non-professional and professional health care workers. Retention of health care professionals is a key challenge not only in developing countries but also within any country in rural and remote areas (94). Imbalance in public and private health sectors also exist. There is also imbalance in gender regarding differences in male and female representation in health care system (95).

CONCLUSION

The practice of PC has now been accepted as the primary mission of pharmacy profession. It has been proved to be very useful in the management of a number of diseases including asthma, hypertension and diabetes. As compared to the developed countries, PC practice has not been satisfactorily implemented in developing countries like Pakistan in both hospital and community pharmacy settings due to certain barriers. Laws exist but are not fully executed due to weak regulatory framework or shortage of sufficient number of pharmacists in hospital as well as community pharmacies. The barriers to pharmaceutical care practice include time constraints, lack of documentation, pharmacist's inadequate training and skills, no-financial support, poor collaboration of health care professionals and wrong perception in people's mind regarding the pharmacists. It is need of time to enhance the public awareness regarding pharmacists and PC practice. In developing countries like Pakistan, imbalance in work health force is found including professional, gender, institutional, and geographical imbalances. Professionals are attracted more towards the urban than the rural areas due to the social, cultural and professional advantages. There is a need to equally distribute the work health force in all the areas of the country to make sure the patients' care and reduced level of morbidity and mortality.

Conflict of interest

There is no conflict of interest among authors over contents of this article.

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ANALYSIS

QUANTIFICATION AND PHARMACOKINETICS OF 1-METHYLPYRIDINIUM AND 1,4-DIMETHYLPYRIDINIUM IN RATS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY. TISSUE DISTRIBUTION OF 1,4-DIMETHYLPYRIDINIUM IN RATS

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Abstract: A sensitive and specific liquid chromatography tandem mass spectrometry method for quantification of 1-methylpyridinium (1-MP) and 1,4-dimethylpyridinium (1,4-DMP) in rat plasma and tissues homogenates was developed. Chromatographic separation was performed on an Aquasil C18 analytical column with an isocratic elution of acetonitrile and water, both with an addition of formic acid (0.1%, v/v). Detection was achieved by triple quadrupole mass spectrometer TSQ Quantum Ultra equipped with a heated electrospray ionization source (HESI). The limit of quantification for both compounds was 0.05 μg/mL in plasma and 0.25 μg/g in studied tissues. The method was applied to pharmacokinetics and bioavailability of both 1-MP and 1,4-DMP with tissue distribution of 1,4-DMP in rats. Pharmacokinetic studies of 1-MP and 1,4-DMP were carried out following their intravenous or intragastric administration to male Wistar rats at the dose of 100 mg/kg. The terminal half-lives of 1-MP and 1,4-DMP after their intravenous administration were 55.3 and 70.8 min, respectively. The absolute bioavailability was 51 and 31% for 1-MP and 1,4-DMP, respectively.

Keywords: LC/MS/MS, method validation, derivatives of pyridinium compounds, pharmacokinetics

Trigonelline, a component of green coffee beans (about 1%) is a product of thermal decomposition, formed during the coffee roasting process. Evaluated compounds: 1-methylpyridinium (1-MP) and 1,4-dimethylpyridinium (1,4-DMP) are the degradation products of trigonelline and for many years they have been a subject of increased interest because of theirs potential hepatoprotective, vasoprotective and antioxidant activity (1-5). Furthermore, some pyridinium salts are known from cytotoxic activity against tumor cells and this effect is probably related to their redox properties (6, 7).

To characterize properties of 1-MP and 1,4-DMP, the structure and surface activity of these compounds were investigated using surface-enhanced Raman spectroscopy (SERS) (8). Recently, liquid chromatography–mass spectrometry method was developed to determine the concentration of 1,4-DMP in rat plasma (9), and this technique was also used for food-derived bioactive pyridines quantification, among them 1-MP and their metabolites in human plasma and urine. The method was applied to monitor the plasma appearance and the urinary excretion, and to calculate the pharmacokinetic parameters of the studied compounds (10, 11). To our knowledge there is no described method for simultaneous determination of 1-MP and 1,4-DMP in complex biological samples, like e.g., tissue homogenates.

The aim of this study was to develop and validate a selective and sensitive bioanalytical LC/MS/MS method for simultaneous quantification of 1-MP and 1,4-DMP in rat plasma and tissue homogenates according to EMA requirements, and finally to assess the pharmacokinetics and bioavail-

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ability of 1-MP and 1,4-DMP considering tissue distribution of 1,4-DMP.

EXPERIMENTAL

Reagents

The 1-methylpyridinium (1-MP) chloride, 1,4-dimethylpyridinium (1,4-DMP) chloride and their stable isotope labeled internal standards: 1-d_3-methylpyridinium 1-MP-d_3) chloride and 1-d_3-methyl-4-methylpyridinium (1,4-DMP-d_3) chloride were provided by dr. J. Adamus from the Institute of Applied Radiation Chemistry, Technical University (Poland). HPLC grade acetonitrile was purchased from J.T. Baker (Germany) and formic acid from Fluka (Germany). Sodium phosphate dibasic, potassium dihydrogen phosphate and sodium chloride were purchased from Sigma-Aldrich (USA). Deionized water was obtained from Millipore system (Direct-Q 3UV, Millipore) and used in all aqueous solutions.

Samples

Plasma and tissues were obtained from adult eight-weeks old male Wistar rats (180-220 g) (Charles River Laboratory, Germany). Rats were injected intraperitoneally with thiopental (60 mg/kg) and blood was collected into heparinized vials after decapitation. The plasma samples were separated by centrifugation (900 × g, 15 min) and stored at -20°C until used. The tissues: liver, lungs, heart, brain, small intestine and kidney were collected, rinsed with phosphate buffer saline solution (PBS, pH = 7.4) and stored at -80°C until used. A piece of thawed tissue was weighted (approximately 100 mg) and homogenized by an UltraTurrax® T10 basic homogenizer (IKA, Germany) in 500 µL of PBS solution (ratio 1 : 5, w/v). The tissue homogenates were prepared directly before the analysis.

Liquid chromatography conditions

The liquid chromatography system UltiMate 3000 (Dionex, USA) consisted of a pump (DGP 3600RS), a column compartment (TCC 3000RS), an autosampler (WPS-3000TRS) and SRD-3600 solvent rack (degasser) was used. Chromatographic separation was carried out on an Aquasil C18 analytical column (4.6 × 150 mm, 5 µm, Thermo Scientific, USA) with the oven temperature set at 30°C. Acetonitrile (A) and water (B), both with a 0.1% (v/v) of formic acid addition were used as mobile phases. Separation of analytes and IS was performed under isocratic condition (A : B; 40 : 60, v/v) at a flow rate of 0.8 mL/min. The autosampler temperature was set at 10°C and the injection volume was 10 µL. The eluent from the HPLC before being directed into the heated electrospray ionization (HESI) probe was split in the proportion of 1 to 2 (1 part to the mass spectrometer and 2 parts to waste). The whole HPLC analysis lasted 10 min.

Mass spectrometric conditions

Mass spectrometric detection was performed on TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, USA) equipped with a HESI II probe operating in the positive ion mode. Quantification was done using selected reaction monitoring (SRM) mode to monitor precursor → product ion transitions of m/z 94 → 79 for 1-MP, m/z 97 → 79 for 1-MP-d_3, m/z 108 → 93 for 1,4-DMP and m/z 111 → 93 for 1,4-DMP-d_3. Data acquisition and processing were accomplished using Xcalibur 2.1 software (Thermo Scientific, USA).

The ion source parameters for all analytes were as follows: ion spray voltage 4000 V, vaporizer temperature 250°C, sheath gas and auxiliary gas (nitrogen) pressure 30 and 10 arbitrary units, respectively, and capillary temperature 350°C. Argon pressure in the collision cell was 1.5 mTorr. Collision energy was set at 23 V for 1-MP, 22 V for 1-MP-d_3 and 30 V for 1,4-DMP and 1,4-DMP-d_3.

Preparation of standard solutions

Stock solutions (1 mg/mL) of 1-MP chloride, 1,4-DMP chloride and its deutered analogs: 1-MP-d_3 chloride and 1,4-DMP-d_3 chloride were individually prepared in ultrapure water. The combined standard solution of 1-MP and 1,4-DMP was prepared by mixing and diluting the appropriate amounts from individual stock solutions. The final concentration of the working standard solutions was 50, 40, 35, 30, 25, 20, 10, 5, 1.5, 1 and 0.5 µg/mL. Internal standard (IS) solution consisted of 1-MP-d_3 and 1,4-DMP-d_3 at a concentration of 25 µg/mL. IS solution was prepared by mixing and diluting the appropriate amounts from individual stock solutions. All stock and working solutions were stored at 4°C until used.

Preparation of calibration and quality control samples

Calibration standards (CC) and quality control samples (QC) were prepared by spiking 10 µL of the appropriate working mixed solution of 1-MP and 1,4-DMP chlorides into 90 µL of blank tissue homogenate or plasma. The concentration of CC points were equivalent to 5, 4, 3, 2, 1, 0.5, 0.1 and 0.05 µg/mL in plasma, and 25, 20, 15, 10, 5, 2.5, 0.5, 0.25 µg/g tissue in tissue samples. Concentration of

QC samples were as follows: limit of quantification (LOQ) at 0.05 µg/mL, low QC (LQC) at 0.15 µg/mL, medium QC (MQC) at 2.5 ng/mL and high QC (HQC) at 3.5 ng/mL in plasma samples, and LOQ at 0.25 µg/g, LQC at 0.75 µg/g, MQC at 12.5 µg/g and HQC at 17.5 µg/g in tissue samples, for both analyzed compounds.

Samples preparation

All analyzed samples were prepared by the way of deproteinization with acetonitrile (12). A 100 µL aliquot of rat plasma or homogenized tissues was pipetted out into a polypropylene tube and spiked with 10 µL of the working IS solution (25 µg/mL). Then, the samples were briefly mixed and 200 µL of acidified acetonitrile (0.1%, v/v) was added. The mixture was again shaken, next, the samples were refrigerated at 4°C for 20 min, and afterwards centrifuged at 16600 × g for 15 min at 10°C. The supernatant (100 µL) was transferred into new tubes and evaporated to dryness at 37°C under a gentle stream of nitrogen gas in a TurboVap evaporator (Caliper Life Sciences, USA). The dry residue was reconstituted with 100 µL of the acetonitrile/water (50/50, v/v) mixture, and an aliquot of 10 µL was injected into the LC/MS/MS system.

Method validation

Method validation was carried out on blank matrices: plasma and brain, liver, heart, kidney, lungs, and small intestine homogenates spiked with an appropriate amounts of 1-MP, 1,4-DMP and their IS following the criteria of bioanalytical method validation (13).

Selectivity/Specificity

The specificity of the method was evaluated by analyzing blank matrices from six different rats. Each blank sample was tested for interferences using the proposed clean up procedure and chromatographic/mass spectrometric conditions.

Accuracy and precision

Precision was calculated in the terms of RSD (%) by analyzing QC samples at four concentration levels of 1-MP and 1,4-DMP (0.05, 0.15, 2.5 and 3.5 µg/mL in plasma, and 0.25, 0.75, 12.5, 17.5 µg/g in the tissues). Accuracy was evaluated as [mean found concentration / theoretical concentration] × 100. The criteria for acceptability of the data included accuracy within ± 15% deviation from the nominal values, and precision within 15% RSD except for LOQ, where it should not exceed ± 20%.

Within day precision and accuracy were executed by repeated analysis (n = 5) of the samples at different QC levels on the same day. Between days precision and accuracy were determined by repeated analysis on the following day. The concentration of each QC sample was determined using the calibration curve prepared and analyzed on the same day.

Matrix effect and extraction recovery

The relative matrix effect was estimated according to Matuszewski (14, 15) by assessing the variability of standard line slopes expressed as a coefficient of variation, RSD (%). The precision values of standard slope lines should not exceed 4% for the method to be considered reliable, and free from the relative matrix effect. For evaluation of the relative matrix effect, five different sources of rat matrices were used.

Extraction recoveries of 1-MP and 1,4-DMP from plasma were determined at LQC and HQC. They were calculated by comparing the mean peak areas obtained for deproteinized QC samples with those of blank extracts with standards added at appropriate concentration which represented the 100% recovery value.

Stability studies

Long-term, short-term, freeze and thaw stability tests were performed for plasma samples. The samples for long-term and short-term stability tests were kept at -20°C for the period of 4 months, and at the room temperature for a period that exceeded the routine sample preparation time (about 5 h), respectively. Post preparation stability test was carried out for all analyzed matrices; samples were stored in autosampler at 10°C for 24 h. A stability study was evaluated using two concentration levels (LQC and HQC). All stability samples were quantified using fresh calibration curve and compared to the nominal concentration in the sample. Samples were considered to be stable if 85-115% of the initial concentration was found.

Pharmacokinetic study in rats

Using the new LC/HESI-MS/MS method, pilot pharmacokinetic studies of 1-MP and 1,4-DMP were carried out following their intravenous or intragastric administration to male eight-weeks old Wistar rats (180-200 g). Rats were kept under conditions of constant temperature (21-25°C), and relative humidity of approximately 40-65% with a standard light/dark cycles. Animals were housed in stainless steel cages with suspended wire-mesh floors (maximum of 5 rats per cage). They were fast-

ed overnight and then weighted. Rats had free access to water throughout the experimental period. Studied compounds: 1-MP and 1,4-DMP, dissolved in 0.9% sterile isotonic saline, at the dose of 100 mg/kg body weight were administered intravenously or intragastrically. Rats were anesthetized *via i.p.* administration of thiopental (60 mg/kg) and sacrificed at the following time intervals: 5, 30, 60, 120 and 240 min after 1-MP or 1,4-DMP intravenous administration, and 10, 30, 60, 120 and 240 min after intragastric dosing. Three rats were sampled at every time point. Blood samples were collected into

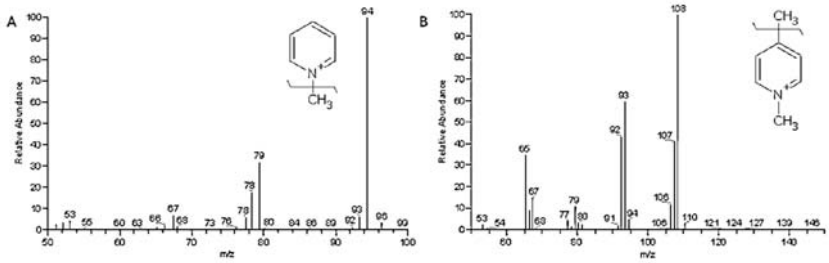

Figure 1. Fragmentation mass spectra of 1-MP (A) and 1,4-DMP (B), collision energy 30 V

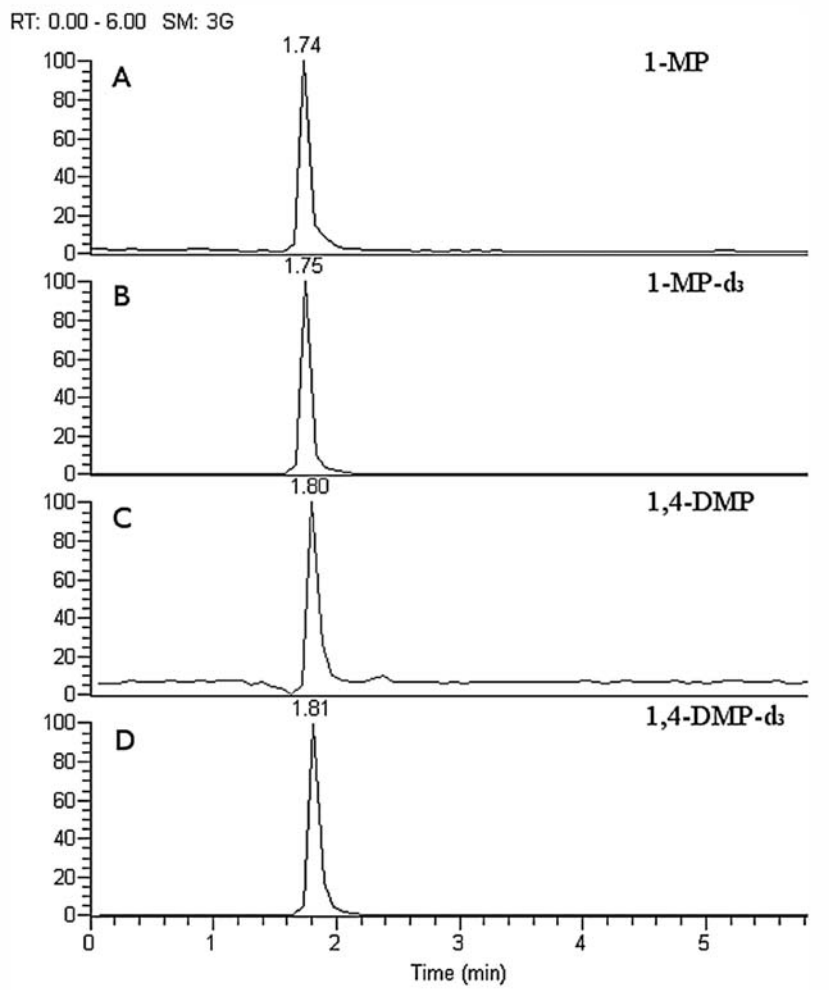

Figure 2. Extracted ion chromatograms of 1-MP (A), 1-MP-d_3 (B), 1,4-DMP (C) and 1,4-DMP-d_3 (D) in rat plasma (LLOQ sample)

microfuge tubes. Plasma and selected tissues (liver, lungs, heart, intestine, brain and kidneys) were stored at –20°C and –80°C until used, respectively.

Pharmacokinetic parameters were calculated by a non-compartmental approach from the average concentration values, using Phoenix WinNonlin software (Certara, USA). First order elimination rate constant (λ_z) was calculated by linear regression of time *versus* log concentration. Next, the area under the mean serum and tissue compound concentration *versus* time curve extrapolated to infinity ($AUC_{0\rightarrow\infty}$) was estimated using the log-linear trapezoidal rule (equation 1), where C_n is the concentration of last sampling of each compound.

Table 1. Relative matrix effect for 1-MP and 1,4-DMP.

Matrix	Lots	1-MP			1,4-DMP		
		Slope	R^2	SD [%]	Slope	R^2	SD [%]
Plasma	1	0.000350	1.0000	3.5	0.000351	0.9999	2.6
	2	0.000318	0.9973		0.000341	1.0000	
	3	0.000330	0.9998		0.000357	0.9995	
	4	0.000335	0.9971		0.000349	0.9998	
	5	0.000329	0.9992		0.000366	0.9990	
Liver	1	0.000407	0.9911	4.1	0.000461	0.9993	2.2
	2	0.000366	0.9981		0.000435	0.9989	
	3	0.000393	0.9999		0.000451	0.9997	
	4	0.000401	0.9941		0.000449	0.9997	
	5	0.000397	0.9967		0.000442	0.9979	
Heart	1	0.000464	0.9977	3.4	0.000449	0.9990	4.0
	2	0.000474	0.9997		0.000423	0.9979	
	3	0.000472	0.9998		0.000425	0.9988	
	4	0.000474	0.9980		0.000411	1.0000	
	5	0.000506	0.9991		0.000404	0.9978	
Lungs	1	0.000605	0.9999	3.3	0.000481	0.9975	2.3
	2	0.000656	0.9998		0.000507	0.9996	
	3	0.000620	0.9961		0.000497	0.9981	
	4	0.000647	0.9988		0.000505	0.9988	
	5	0.000639	0.9981		0.000511	0.9979	
Kidney	1	0.000572	0.9959	2.1	0.000484	0.9985	2.1
	2	0.000576	0.9996		0.000462	0.9999	
	3	0.000591	0.9994		0.000476	0.9985	
	4	0.000558	0.9993		0.000462	0.9998	
	5	0.000568	0.9997		0.000465	0.9996	
Brain	1	0.000581	0.9971	3.7	0.000502	0.9993	3.7
	2	0.000569	1.0000		0.000486	0.9989	
	3	0.000550	0.9984		0.000487	0.9994	
	4	0.000605	0.9966		0.000530	0.9967	
	5	0.000560	0.9996		0.000491	0.9992	
Intestine	1	0.000285	0.9989	3.6	0.000285	0.9972	3.6
	2	0.000288	1.0000		0.000288	0.9976	
	3	0.000306	0.9997		0.000306	0.9996	
	4	0.000278	0.9991		0.000278	0.9996	
	5	0.000284	0.9999		0.000284	0.9993	

Table 2. Within day accuracy (% of nominal concentration) and precision (% RSD) for 1-MP and 1,4-DMP in plasma and rat tissues (n = 5).

Matrix	QC level	1-MP			1,4-DMP		
		Mean concentration	Accuracy [%]	Precision RSD [%]	Mean concentration	Accuracy [%]	Precision RSD [%]
Plasma[a]	LLQC	0.053	106.6	7.9	0.050	100.2	1.4
	LQC	0.140	93.2	4.6	0.140	93.1	3.1
	MQC	2.539	101.6	3.2	2.434	97.4	3.8
	HQC	3.365	96.1	8.4	3.423	97.8	5.0
Liver[b]	LLQC	0.238	95.0	12.9	0.254	101.4	3.4
	LQC	0.678	90.3	3.6	0.668	89.0	0.6
	MQC	11.347	90.8	6.3	11.637	93.1	1.2
	HQC	16.497	94.3	4.7	16.658	95.2	6.0
Heart[b]	LLQC	0.225	89.8	3.8	0.260	104.0	6.9
	LQC	0.739	98.6	8.8	0.730	97.3	4.4
	MQC	12.667	101.3	3.9	12.699	101.6	1.8
	HQC	17.715	101.2	2.4	17.496	100.0	1.8
Lungs[b]	LLQC	0.242	96.7	12.3	0.238	94.9	6.0
	LQC	0.693	92.3	6.2	0.690	91.9	3.5
	MQC	11.853	94.8	6.8	11.805	94.4	2.8
	HQC	16.127	92.2	4.7	16.120	92.1	2.5
Kidney[b]	LLQC	0.288	115.0	2.9	0.237	94.8	5.4
	LQC	0.795	106.0	1.9	0.753	100.4	5.4
	MQC	13.832	110.7	1.4	13.590	108.7	1.1
	HQC	18.974	108.4	3.4	18.693	106.8	3.8
Brain[b]	LLQC	0.242	96.7	9.3	0.248	99.1	15.4
	LQC	0.750	100.0	5.4	0.779	103.8	8.9
	MQC	13.087	104.7	2.2	13.303	106.4	3.3
	HQC	17.289	98.8	1.9	17.882	102.2	3.2
Intestine[b]	LLQC	0.245	97.8	5.8	0.206	82.3	0.2
	LQC	0.679	90.6	2.4	0.700	93.3	5.7
	MQC	12.181	97.4	4.2	13.249	106.0	4.9
	HQC	16.419	93.8	3.1	17.943	102.5	7.8

[a] Mean concentration [µg/mL], [b] mean concentration [µg/g].

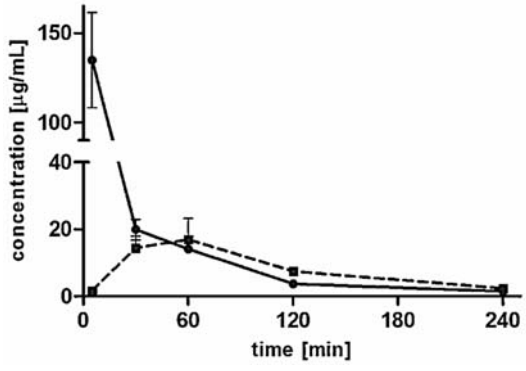

Figure 3. Plasma concentration-time profile of 1-MP after a single intravenous (●) or intragastric (■) administration to rats at the dose of 100 mg/kg

Table 3. Accuracy (% of nominal concentration) and precision (%RSD) between days for 1-MP and 1,4-DMP in plasma and rat tissues.

Matrix	QC level	1-MP			1,4-DMP		
		Mean concentration	Accuracy [%]	Precision RSD [%]	Mean concentration	Accuracy [%]	Precision RSD [%]
Plasma[a]	LLQC	0.051	102.2	8.6	0.0499	99.7	5.0
	LQC	0.148.	99.1	7.6	0.150	100.2	8.1
	MQC	2.607	104.3	4.8	2.574	103.0	7.2
	HQC	3.531	100.9	10.1	3.597	102.8	7.6
Liver[b]	LLQC	0.235	94.1	9.2	0.262	104.6	5.8
	LQC	0.720	96.0	9.6	0.675	90.0	2.8
	MQC	11.879	95.0	8.5	11.945	95.6	4.5
	HQC	16.324	93.3	5.1	17.014	97.2	4.6
Heart[b]	LLQC	0.197	94.6	11.3	0.265	105.8	6.4
	LQC	0.661	101.8	8.4	0.737	98.2	5.1
	MQC	10.202	103.7	3.8	12.902	103.2	3.0
	HQC	17.159	102.6	4.4	17.630	100.7	4.4
Lungs[b]	LLQC	0.252	100.5	14.4	0.249	99.4	6.2
	LQC	0.733	97.7	6.9	0.738	98.3	9.2
	MQC	12.442	99.5	7.3	12.799	102.4	8.8
	HQC	16.986	97.1	6.6	17.292	98.8	7.9
Kidney[b]	LLQC	51.147	102.3	13.58	0.245	97.8	7.5
	LQC	157.860	105.2	6.21	0.780	104.0	5.8
	MQC	2752.947	110.1	8.03	13.401	107.2	3.2
	HQC	3654.619	104.4	4.95	18.297	104.6	3.5
Brain[b]	LLQC	0.248	99.3	12.5	0.236	94.3	10.2
	LQC	0.751	100.1	4.9	0.745	99.3	7.9
	MQC	13.043	104.3	3.2	12.945	103.6	4.5
	HQC	17.704	101.2	5.3	17.897	102.3	4.9
Intestine[b]	LLQC	0.260	104.0	8.8	0.239	95.3	16.8
	LQC	0.721	96.1	7.4	0.743	99.0	9.0
	MQC	12.356	98.8	3.5	12.619	100.9	7.5
	HQC	16.932	96.8	4.6	17.097	97.7	8.8

[a] Mean concentration [µg/mL], [b] mean concentration [µg/g].

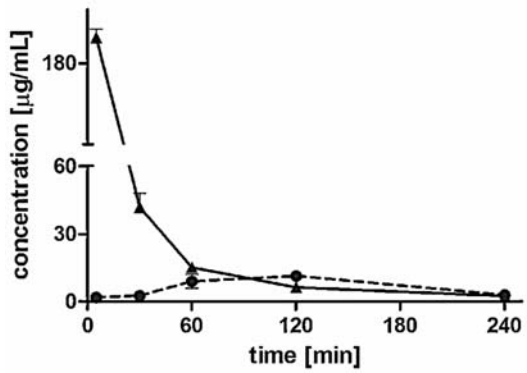

Figure 4. Plasma concentration-time profile of 1,4-DMP after a single intravenous (●) or intragastric (■) administration to rats at the dose of 100 mg/kg

Table 4. Stability for 1-MP and 1,4-DMP in rat plasma.

QC level	1-MP			1,4-DMP		
	Mean concentration (µg/mL)	Accuracy [%]	Precision RSD [%]	Mean concentration (µg/mL)	Accuracy [%]	Precision RSD [%]
Short-term stability test						
LQC	0.1463	97.5	8.7	0.151	100.6	1.5
HQC	3.135	89.6	5.7	3.141	89.7	7.5
Freeze and thaw stability test						
LQC	0.1589	105.9	4.2	0.1483	98.9	6.8
HQC	3.900	111.4	2.7	3.372	96.3	3.4
Long-term stability test						
LQC	0.1528	101.9	4.3	0.130	86.7	8.6
HQC	3.383	96.6	1.1	3.200	91.4	1.6

Table 5. Post-preparative stability for 1-MP and 1,4-DMP.

Matrix	QC level	1-MP			1,4-DMP		
		Mean concentration	Accuracy [%]	Precision RSD [%]	Mean concentration	Accuracy [%]	Precision RSD [%]
Plasma[a]	LQC	0.162	108.2	10.9	0.156	103.7	4.2
	HQC	3.557	101.6	3.3	3.647	104.2	2.8
Liver[b]	LQC	0.913	122	6.2	0.658	87.7	4.1
	HQC	16.664	95	4.7	15.629	89.3	3.6
Heart[b]	LQC	0.606	80.8	10.9	0.667	88.9	3.7
	HQC	15.731	89.9	6.0	16.081	91.9	3.2
Lungs[b]	LQC	0.789	105.2	9.1	0.919	122.5	5.0
	HQC	17.779	101.6	4.	19.362	110.6	3.4
Kidney[b]	LQC	1.012	135	3.2	0.740	98.6	6.1
	HQC	18.703	107	2.4	18.388	105.1	4.7
Brain[b]	LQC	0.793	105.7	3.9	0.727	96.9	9.0
	HQC	17.161	98.1	2.7	18.147	103.7	4.9
Intestine[b]	LQC	0.768	102.4	5.7	0.963	128.4	9.3
	HQC	17.618	100.7	3.2	18.629	106.4	8.1

[a] Mean concentration [µg/mL], [b] mean concentration [µg/g].

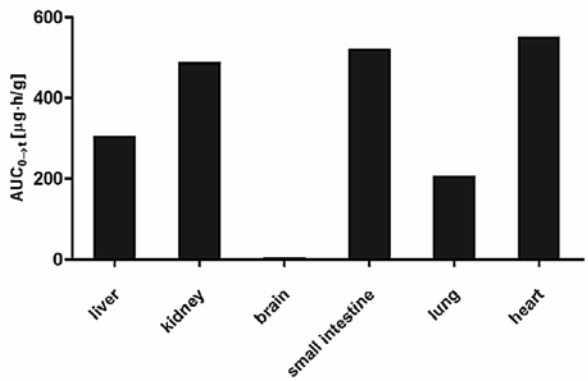

Figure 5. Tissue distribution of 1,4-DMP after a single intravenous administration of compound at the single dose of 100 mg/kg in rats

Table 6. Pharmacokinetic parameters for 1,4-DMP and 1-MP after a single intravenous and intragastric administration at the dose of 100 mg/kg in rats.

Pharmacokinetics parameters	1,4-DMP		1-MP	
	Intravenous	Intragastric	Intravenous	Intragastric
$AUC_{0\to\infty}$ [μg·min/mL]	6.284	1.944	4.245	2.159
MRT [min]	44.34	140.28	38.81	110.58
$t_{1/2}$ [min]	70.77	60.41	55.32	63.26
C_{max} [μg/mL]	-	11.38	-	16.91
V_{ss} [mL/kg]	706	-	914	-
Cl [mL/min/kg]	15.91	-	23.56	-
F [%]	-	31		51

Table 7. Pharmacokinetic parameters in rat tissues after a single intravenous administration of 1,4-DMP at the dose of 100 mg/kg.

Tissue	Pharmacokinetic parameters			
	$AUC_{0\to\infty}$ [μg·min/g]	C_{max} [μg/g]	t_{max} [min]	MRT [min]
Liver	305.03	243.80	30	115
Kidney	488.70	584.45	5	70
Brain	4.87	3.42	5	244
Intestine	530.16	232.92	5	157
Lungs	210.67	115.22	5	143
Heart	551.47	162.80	120	292

$$AUC_{0\to\infty} = \sum_{i=1}^{n}([c_i + c_{i+1}]/2) \cdot (t_{i+1} - t_i) + c_n/\lambda_z \quad (1)$$

Area under the first-moment curve ($AUMC_{0\to\infty}$) was estimated by calculation of the total area under the first-moment curve and extrapolated area using the equation 2, where t_n is the time of last sampling.

$$AUMC_{0\to\infty} = \sum_{i=1}^{n}((t_i \cdot c_i + t_{i+1} \cdot c_{i+1})/2) \cdot (t_{i+1} - t_i) + (t_n \cdot c_n)\lambda_z + c_n/\lambda_z 2 \quad (2)$$

Mean residence time (MRT) was calculated as:

$$MRT = AUMC_{0\to\infty}/AUC_{0\to\infty} \quad (3)$$

Systemic clearance (Cl) was calculated as:

$$Cl = D_{iv}/AUC_{0\to\infty} \quad (4)$$

Volume of distribution at steady state (V_{ss}) was calculated as:

$$V_{ss} = \frac{D_{iv} \cdot AUMC_{0\to\infty}}{(AUC_{0\to\infty})^2} \quad (5)$$

where D_{iv} is an intravenous dose of studied compound, AUMC is the area under the first moment curve, and AUC is the area under the zero moment curve.

The absolute bioavailability (F) after the extravascular (*e.v.*) administration compared to the intravenous (*i.v.*) route was calculated as follows:

$$F = \frac{AUC_{e.v.}}{AUC_{i.v.}} \quad (6)$$

RESULTS AND DISCUSSION

Validation data

The newly developed bioanalytical method for the simultaneous analysis of 1-MP and 1,4-DMP in rat matrices (plasma and selected tissue samples) using LC/HESI-MS/MS technique was developed and validated in the first step of the study.

In order to construct the appropriate SRM method, the most abundant parent and fragmentation ions of analyzed compounds and IS were chosen (Fig. 1). Representative chromatograms from rat plasma samples are shown in Figure 2. Retention times are around 1.7 and 1.8 min for 1-MP and 1,4-DMP, respectively. No significant interferences with other endogenous molecules in sample were observed.

The obtained limit of detection for both compounds was high and equaled 0.01 μg/mL and 0.05 μg/g in plasma and tissues, respectively. It provides the measurement of studied analogs' concentration

in the biological samples from pharmacokinetic experiments. The obtained results, in all matrices, show good linearity over the entire concentration range 0.05-5 µg/mL for plasma and 0.25-25 µg/g for tissues. Calibration curves were generated using weighted (1/x) linear regression analysis. The extraction recoveries of 1-MP and 1,4-DMP in rat plasma were 87.8 ± 8.2% and 92.9 ± 9.8%, respectively. No relative matrix effect for studied compounds (Table 1) was observed for five different tested plasma lots, what can indicate, that developed method is reliable and can be used in routine laboratory work.

Precision was evaluated as repeatability (within day precision) and reproducibility (between days precision). The accuracy and precision results for all matrices are shown in Table 2 (within day) and Table 3 (between days). The obtained results were within the acceptable limits established by EMA for bioanalytical methods (13) confirming that the method can be used for quantifying both 1-MP and 1,4-DMP compounds in the following rat tissues: liver, lungs, heart, brain, small intestine, kidneys and plasma.

The stability of analytes in rat plasma was investigated under a variety of storage and process conditions described in a previous section. Compounds showed to be stable during storage under various conditions (Table 4). Results of post-preparative stability (24 h) for all tested matrices are shown in Table 5. Moreover, results showed, that both 1-MP and 1,4-DMP were stable in all analyzed matrices, but not in liver and kidney. Since, prepared samples of liver and kidney for 1-MP, and in case of small intestine and lungs for 1,4-DMP should be analyzed within 24 h. The validated LC/HESI-MS/MS method was successfully used for quantification of 1-MP and 1,4-DMP in rat plasma following theirs intravenous or intragastric administration at a dose of 100 mg/kg. The concentration of 1,4-DMP in selected tissues were also determined with desired accuracy and precision.

Pharmacokinetic study in rats

Blood samples during experiments were collected in a regular time intervals. The mean concentration – time profiles of 1-MP and 1,4-DMP in plasma were plotted in Figures 3 and 4, respectively.

The results of the model independent pharmacokinetic data analysis obtained following intravenous or intragastric administration of 1-MP or 1,4-DMP in plasma are summarized in Table 6. Target analogs were eliminated rather slowly with the terminal half-life times for 1-MP equaled 55.3 min, and for 1,4-DMP equaled 70.8 min, after their intravenous administration. The volumes of distribution at the steady-state were in the range of 0.9 L/kg and 0.7 L/kg for 1-MP and 1,4-DMP, respectively, and might indicate theirs intracellular disposition. The absolute bioavailability estimated based on the $AUC_{0\rightarrow\infty}$ calculated from time zero to infinity yielded the values of 51% for 1-MP and 31% for 1,4-DMP, and was rather low, with the peak concentration occurring 60 min for 1-MP, and 120 min for 1,4-DMP after their intragastric administration.

1,4-DMP has significant tissue distribution which is in agreement with its high volume of distribution (0.7 L/kg). Analysis showed that the highest amount of 1,4-DMP was observed in heart, then in kidney and small intestine, and the lowest one was detected in brain (Fig. 5). Distribution was rapid and the maximal concentration occurred in the most of tissues at 5 min after administration with exception of liver (maximum at 30 min) and heart (maximum at 120 min) as seen in Table 7. These findings suggest that 1,4-DMP is mostly distributed in heart and, despite the fact that this compound is positively charged in physiological pH, it can penetrate blood-brain barrier, probably *via* a specific carrier system.

CONCLUSIONS

A rapid and simple LC/HESI-MS/MS method was developed and validated for quantification of 1-MP and 1,4-DMP in plasma and selected rat tissues. The assay showed wide linear dynamic range of 0.05–5 µg/mL for plasma, and 0.25–25 µg/g for tissues with acceptable within day and between days accuracy and precision. The method was successfully applied to assess the pharmacokinetic profiles of 1-MP and 1,4-DMP in rats after compounds intravenous and intragastric administration. The absolute bioavailability in rats was estimated at 51% for 1-MP and 31% for 1,4-DMP, respectively. The distribution of 1,4-DMP in tissues was rapid with the maximal concentration occurred at 5 min after compound administration in kidney, intestine, lungs and brain.

Acknowledgment

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DETERMINATION OF TENOXICAM IN THE PLASMA BY REVERSE PHASE HPLC METHOD USING SINGLE STEP EXTRACTION TECHNIQUE: A RELIABLE AND COST EFFECTIVE APPROACH

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Abstract: A simple and cost effective RPLC-UV bio-analytical method was developed and used for tenoxicam quantification on ODS Hypersil C-18 column using classical liquid-liquid extraction technique for sample preparation. Acetonitrile was used as precipitating agent for plasma proteins and supernatant was taken for injection without any further modification. The bio-analytical method depends upon isocratic elution using binary mixture of aqueous 0.1 M potassium dihydrogen phosphate and acetonitrile in 6 : 4 ratio. The pH of mobile phase was adjusted to 2.8 which favor tenoxicam to remain undissociated throughout the analysis. The optimized flow rate of 1.0 mL/min provided proper separation of peaks and column clean up within 5 min. The UV detection was achieved at 381 nm and 4.29 min. Reproducible calibration curve gave 0.325 µg/mL LOQ, linear dynamic range from 0.325 to 20 µg/mL and recovery from plasma was 98.5% with %CV 0.2314 achieved. After validation, the method was applied in pharmacokinetic study in healthy human volunteers (n = 8). The pharmacokinetic parameters were evaluated using kinetica version 4.1.1. The values of C_{max} and area under curve for current study were 1.776 ± 0.003 µg/mL and 179.97 ± 0.0681 (mean ± SEM) µg × h/mL. The values of $t_{1/2}$ and volume of distribution for tenoxicam in current study were 74.103 ± 0.167 h (mean ± SEM) and 11.962 ± 0.0677 L/kg (mean ± SEM), respectively. This method was simple, sensitive and successfully applied in pharmacokinetic studies. It can be extended to bioequivalence studies and evaluation of tenoxicam in different clinical situations.

Keywords: cost-effective, simple, HPLC, tenoxicam, pharmacokinetics

Non-steroidal anti-inflammatory drugs (NSAIDs) include most of the over the counter (OTC) medicines which are reported for about 70 million prescriptions and 30 billion OTC medications being sold annually in America (1, 2). NSAIDs represent a family of drugs which exhibits potent antipyretic, analgesic and anti-inflammatory activity. These drugs mainly include oxicam family, N-phenyl anthranilic acid derivatives and carboxylic acid derivatives used frequently in painful and inflammatory conditions (3, 4).

Tenoxicam, a thienothiazine derivative NSAID (Fig. 1) belongs to enolic acid group (oxicam family) and is prescribed for its analgesic, anti-pyretic and anti-inflammatory effects, resulting from inhibition of cyclo-oxygenase enzymes and subsequent formation of prostaglandins in musculoskeletal disorders (5-7).

Tenoxicam (TX), when given orally exhibits complete absorption with 99% plasma protein binding and low volume of distribution (Vd) (0.12 to 0.15 L/kg) (8). TX presents maximum plasma concentration (C_{max}) range from 1.7 to 3.6 µg/mL after single dose of 20 mg taken orally and extended half-life ($t_{1/2}$) of 60-75 h, which allows once daily administration (9-12). Liver provides major catabolic

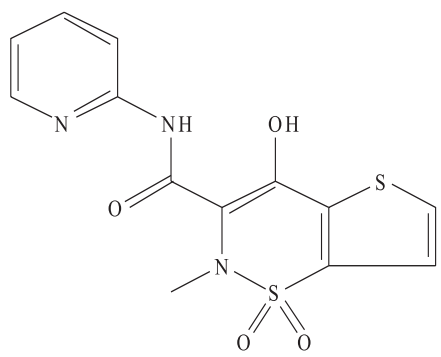

Figure 1. 2D structure of tenoxicam

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pathway and TX is excreted out as inactive metabolites (mainly 5'-hydroxy-tenoxicam) in feces and urine with less than 1% in unchanged form (8, 13).

2-Aminopyridine (2AP), a synthetic precursor or decomposition product is major impurity found in bulk as well as oxicams formulations (14). Associated side effects (such as depression and nervousness) with TX in long term management of rheumatic conditions can lead to non-compliance and discontinuation of the therapy (15). Pharmacokinetics of TX is well established but it still needs to be studied in local populations as physiological differences among populations affect pharmacokinetics (16, 17). This demands validated analytical method for TX measurement both in bulk and in plasma.

Various HPLC methods have been developed for TX determination in bulk or in bulk and plasma. These methods differ in sample preparation and mobile phase composition (16). Some studies involved liquid-liquid extraction with or without evaporation while others employed solid phase extraction (SPE). Use of different cartridges makes solid phase extraction an expensive analytical method while liquid-liquid extraction with evaporation makes the procedure tedious with fewer turnovers. Although different studies utilized liquid-liquid extraction without evaporation, they were hindered by complex nature of mobile phase (16, 18).

HPLC tandem mass spectrometry method for oxicam analysis using mixture of acetonitrile and 10 mM ammonium phosphate buffer as mobile phase in 50 : 50 ratio has been reported. However, this method involves sample preparation using cartridge columns and vacuum with washing of column using different solvents for activation of column and drug extraction. It also involved sample drying with nitrogen and then reconstitution. It makes overall sample preparation tedious (19).

A UHPLC method with tandem mass spectrometry for a range of NSAIDs has been described which involves gradient elution and varying the composition of mobile phase frequently. It also involves use of different cartridges and solvents for sample preparation making the analysis hectic (20).

Sora et al. reported fast RP-HPLC UV method with submicron column using gradient elution. But mobile composition was varied to 100% organic phase with flow rate up to 2.0 mL/min making method costly. The sample preparation was done using precipitation method but the recovery is lower than in present study (16).

The present study uses liquid-liquid extraction without evaporation and binary mobile phase of phosphate buffer and acetonitrile. The low LOQ (limit of quantification) as in this study was considered satisfactory for pharmacokinetic evaluation as plasma levels of TX are high enough. This study provides a validated, simple and cost effective HPLC method for TX analysis in plasma with sufficient sensitivity.

EXPERIMENTAL

Instrumentation

Experimental work was performed with Sykum s 2100 solvent delivery system, Sykum s

Table 1. Percent recovery of tenoxicam against spiked plasma.

Tenoxicam (actual conc. µg/mL)	Percent recovery (%)	%CV
0.325	98.764	0.638
2.5	98.152	0.239
20	99.126	0.262
Mean	98.68	0.379

Table 2. Intra and intra batch precision studies of TX in spiked plasma.

Spiked concentration (ng)	Intra batch n = 9		Inter batch n = 9	
	Mean ± SD	%CV	Mean ± SD	%CV
325	317.0 ± 2.0	0.6309	316.22 ± 3.73	1.181
2500	2310 ± 60.0	2.5974	2288 ± 85.0	3.69
20000	18433 ± 472	2.5637	19796 ± 80.0	0.403

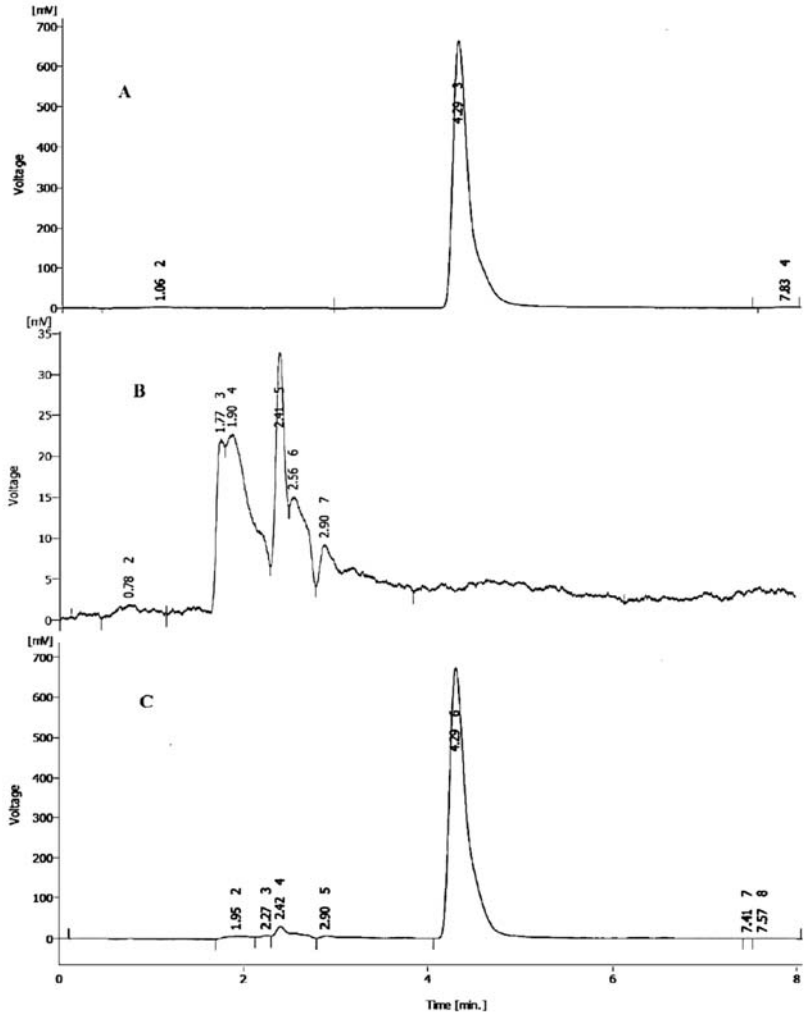

Figure 2. Chromatograms: (A) aliquot (20 µg/mL) of drug standard solution, (B) blank plasma, (C) spiked plasma (20 µg/mL)

4011 column thermo controller and Sykum s 3210 UV-Vis detector. System control and acquisition of data were carried out with clarity software version 2.5.6.99. Determination of λ_{max} of drug in given solvent system was performed with UV-Vis spectrophotometer (U2020), IRMECO, Japan.

Materials

Ortho phosphoric acid, potassium dihydrogen phosphate, HPLC grade methanol and acetonitrile were purchased from Merck KGaA, Germany. HPLC water (deionized double distilled) was prepared with Irmeco GmbH, Geesthacht, Germany apparatus within laboratory. TX was gifted by Fynk Pharmaceuticals (Pvt.) Ltd., Lahore, Pakistan. Plasma was provided by Blood Bank and Thalassemia Center, Bahawal Victoria Hospital, Bahawalpur, Pakistan.

Sample preparation and plasma treatment

Tenoxicam powder (50 mg) was weighed and dissolved in 50 mL of mobile phase to make stock solutions from which serial dilutions were prepared to cover 0.320-20 ug/mL range. Peak area to concentration ratio was used for calibration curve and regression equation derivations.

Human plasma was spiked with equal volume of drug aliquots, vortexed and same amount of acetonitrile (precipitating agent) was added. Mixture was vortexed again and centrifuged at 14000 rpm for 10 min. The supernatant layer was taken and filtered through microfiltration assembly before injecting.

Chromatographic method

Method development, validation and pharmacokinetic studies were performed in ambient condi-

tions on ODS Hypersil C18 column (250 × 4.6 mm dimension and 5 mm particle size) with isocratic elution using aqueous 0.1 M potassium dihydrogen phosphate buffer and acetonitrile in 6 : 4, v/v ratio as mobile phase. The pH of mobile phase was adjusted to 2.8 (using ortho-phosphoric acid), to favor undissociated elution of TX. The optimized flow rate 1.0 mL/min was maintained throughout analysis with UV detection at 381 nm.

The goals achieved by the application of such chromatographic conditions are appropriate retention of drug providing separation of plasma and TX peaks and column equilibration within 5 min period.

Pharmacokinetic application

Pharmacokinetic study of TX was performed consisting of fast state, one treatment of 20 mg single dose taken orally. Healthy volunteers (n = 8), not taking any medication for last three weeks, were included in the study. Standard meal was given after 2 h of drug administration. Samples of venous blood were collected in EDTA tubes at (0) for pre-dose, and then at 3, 6, 12, 18, 24, 36, 48, 60, 72, 96, 120, 144, and 168 h for post-dsose. The samples were mixed, centrifuged at 4000 rpm for 10 min and supernatant layer containing plasma was collected and stored at ultra-low temperature. Supernatant, obtained from centrifugation of equivolume mixture of plasma and acetonitrile at 14000 rpm, was injected after filtration through microfiltration assembly.

Non-compartmental model approach was used for pharmacokinetic analysis using "Kinetica" version 4.4.1 and "Microsoft excel". Pharmacokinetic parameters including area under curve (AUC), maximum plasma concentration (C_{max}), half-life, volume of distribution and clearance were calculated by the same software.

RESULTS AND DISCUSSION

Recovery studies of TX

Recovery studies were performed in triplicate with lower, medium and high concentrations of drug for evaluation of any effect of medium on drug.

Table 3. Bench top stability studies.

Statistics	Cycle							
	0 h		2 h		4 h		6 h	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Mean ± SD	0.3130 ± 0.004	19.133 ± 0.208	0.30 ± 0.0012	19.040 ± 0.152	0.2990 ± 0.002	18.830 ± 0.252	0.290 ± 0.0025	18.90 ± 0.424
%CV	1.2926	1.0880	0.3845	0.7972	0.6970	1.3363	0.8698	2.2448

Table 4. Freeze thaw stability studies.

Statistics	Cycle							
	Cycle 0		Cycle 1		Cycle 2		Cycle 3	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
Mean ± SD	0.320 ± 0.002	19.690 ± 0.121	0.320 ± 0.002	19.50 ± 0.121	0.310 ± 0.003	19.350 ± 0.042	0.310 ± 0.005	19.40 ± 0.031
%CV	0.541	0.612	0.481	0.620	0.801	0.215	1.478	0.157

Table 5. Pharmacokinetic parameters of tenoxicam.

Statistics	C_{max}[a]	AUC[b]	Vd[c]	Cl[d]	$t_{1/2}$[e]
Mean	1.776	179.97	11.962	0.111	74.103
SD	± 0.012	± 2.58	± 0.662	± 0.002	± 4.075
SEM	0.003	0.068	0.0677	0.002	0.167

[a]Peak plasma concentration. [b]Area under the curve. [c]Volume of distribution. [d]Clearance. [e]Half life

The recovery of TX against spiked plasma is greater than 98% with %CV of 0.379% using acetonitrile as precipitating agent in acidic pH.

Drug interaction with plasma proteins lowers recovery but protein precipitation with acetonitrile appeared effective.

Sensitivity

Sensitivity is the capability of analytical method to measure smaller concentration of analyte, which is explained on the basis of limit of detection (LOD) and limit of quantification (LOQ). LOD is the smallest concentration of analyte which gives a 3 : 1 of signal to noise ratio and is not necessarily quantified. LOQ is considered generally 3 times higher than LOD and it can be quantified. The method provides enough sensitivity with LOD 100 ng/mL and LOQ 325 ng/mL.

Robustness

The robustness studies are aimed for reproducibility of results and in expressing the repeatability of results when method's inherent or external factors were varied. The ambient temperature fluctuations have no effects on retention parameters. Reduction in buffer strength in mobile phase by less than 20% or doubling does not affect the reproducibility of results. The method was also run on Agilent 1100 series HPLC with DAD detector, which proved the robustness of method.

Linearity and calibration

A curve obtained by response of an instrument upon different concentrations of analyte within linear response range is called standard/calibration curve and it demonstrates the relationship between introduced concentration and concentration back calculated.

While constructing standard curve for all dilutions, within the range of liner response 325 ng - 20 µg, the measurements were run in triplicate and parameters of standard curve were calculated from the data. The computed values of standard curve parameters are 525.42, 113.66 and 0.9976 for slope, intercept and r^2, respectively.

Precision

Precision explains if any random error is affecting the results of method and is expressed as percent coefficient of variation (%CV), standard deviation (SD), relative standard deviation (RSD) and variance (S^2). Precision study comprised triplicate run of lower, middle and higher strengths both for inter batch and intra batch and results are expressed as %CV in Table 2.

For the assessment of accuracy of the method three concentrations from the same and three from different batches were run in triplicate as lowest, middle and highest within same day at different times and at different days.

The study results are expressed in percentage agreement with reference for intra batch accuracy as 98.64% for higher, 98.36% for lower and 99.06% for medium concentrations. For inter batch for higher, medium and lower concentrations the results were 98.90, 99.59 and 98.44%, correspondingly.

Stability studies

Stability studies of TX were carried out in spiked plasma to investigate whether the drug remains stable for sufficient time before the analysis is performed.

Bench top studies were performed to evaluate the stability of drug in solvent in which drug is dissolved. These studies were conducted by storing lower quality control (LQC) and higher quality control (HQC) samples at ambient temperature and then run at different time intervals of 0, 2, 4, and 6 h. The results are expressed in %CV in Table 3.

Freeze thaw studies include lower and higher quality control investigations, conducted in four cycles. The samples were taken at 0, 1, 2 and 3 cycles. The %CV expresses the results of study.

Pharmacokinetic studies

The bioavailability and pharmacokinetic parameters were computed from the concentrations of the drug in different plasma samples of volunteers and given in Table 5.

A range of maximum plasma concentration from 2 to 4 µg/mL are described after receiving 20-40 mg tablet of TX orally (13). Another report suggested even a broader range from 1.7-3.6 µg/mL of peak plasma concentration ($C_{max)}$ for TX after taking 20 mg orally (9). The area under the curve (AUC) is lower than described in the literature and other parameters are in agreement with previously reported (16, 21). This variation in C_{max} can be explained on the basis of population factor, as populations of different regions have physiological differences, different psychological states and dietary habits. Studies have shown that bioavailability of TX decreases with an increase of pH in stomach whether by taking antacids or by other factor (22).

CONCLUSION

A simple and economical RP-HPLC method was developed for the analysis of TX. This method

was simple involving less components for mobile phase formulation and sample preparation.

Sample preparation applies liquid-liquid extraction with acetonitrile as precipitating agent and chromatographic separation is carried on ODS Hypersil column at ambient temperature. The flow rate was maintained at 1.0 mL/min, which provides high resolution and rapid column re-equilibration. Detection of analyte was performed at 381 nm in UV detector, so the selectivity of method was increased.

The developed method was validated for different parameters of its precision, reproducibility and stability studies of analyte. Then, this method was applied for conducting *in vivo* studies of TX in healthy human volunteers. Pharmacokinetic studies of TX were performed and pharmacokinetic parameters were determined. Simplicity and cost effectiveness are the advantages of the method. The applications of this method can be extended to clinical situations and bio-equivalence studies.

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DRUG BIOCHEMISTRY

THE EFFECT OF METHANOL USED AS VEHICULUM ON SERUM PHENACETIN CONCENTRATION IN THE RAT

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Abstract: The xenobiotic absorption process is dependent on many factors, related both to the substance and form of its administration. During administration of small amounts of drugs, the effect of vehiculum on drug fate in the body becomes also evident. The intensity of absorption depends on numerous factors not necessarily related to the substance and its formulation, and also on biotransformation and active transport processes. Additional problem is the fact that many medicines are lipophilic compounds and insoluble in the water (e.g. phenacetin). Methanol and its aqueous solutions facilitate administration to the experimental animals, in the dissolved form of a number of medicines practically insoluble in water. Taking into consideration that methanol is particularly for rats, of low toxicity, it is quite frequently applied as vehiculum. The aim of this study was to investigate the potential interactions that may occur during the use of methanol as vehiculum and compare changes when were used solution 1% of carboxymethylcellulose. The study was performed on male Wistar rats. The tests were performed using phenacetin, which is recognized as biomarker of CYP 2E1 isoform activity. Phenacetin was given per os in a single dose of 100 mg/kg b. w. Various procedures of phenacetin administration were tested, including solubilization in methanol or suspension in 1% water solution of carboxymethylcellulose. The results of this study show that methanol influences the phenacetin bioavailability and kinetics. Comparing the administration of this drug in methanol solutions against 1% of carboxymethylcellulose, it is in the case of phenacetin triple increase in $AUC_{0\text{-}4\ h}$. The presence of methanol affects the shape of kinetic curves of phenacetin causing higher their course until 4 hours after administration.

Keywords: vehiculum, methanol, carboxymethylcellulose, phenacetin, acetaminophen, metabolism, bioavailability, rats

Abreviations: ADH - alcohol dehydrogenase system, AUC - area under the concentration-time curve, CMC - 1% aqueous solution of sodium carboxymethylcellulose, MetOH – 100% methanol, P-gp - P-glycoprotein, PHE - phenacetin

Administration of drugs and other xenobiotics is inevitably connected with the selection of an ideal vehiculum that should facilitate drug absorption, but not interfere with the action of an investigated compound. Our attention was focused on methanol since many water-insoluble xenobiotics can be dissolved in methanol or water-methanol solutions.

Methanol is rapidly distributed to organs according to the distribution of body water. The catalase-peroxidase system and the alcohol dehydrogenase system (ADH) are major enzymes involved in methanol biotransformation (1, 2) (Fig. 1).

In rats, the oxidation of methanol is performed primarily by catalase. In rodents, metabolism is fast enough to avoid accumulation of formic acid so that the toxicity of methanol in these animals is relatively low (2).

In vitro studies of human liver microsomes have shown that methanol inhibits activity of cytochrome P450 1A2, 2E1, and 2C9 (3). It is likely that this effect also occurs *in vivo*. In addition, the interaction of solvents with active transport by glycoprotein P (P-gp) and other transporters could be found.

In studies conducted *in vitro* on human microsomes (3, 4), human lymphoblastic cells (5) and rat microsomes (6) many researchers showed inhibitory effect of methanol on the CYP 1A2. They claimed

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that the effect of methanol is concentration dependent. Already a concentration of 1% inhibit cytochromes activity, while in 5% concentration of this solvent they observed significant inhibition of multiple cytochrome isoforms. There was not studied the effect of this solvent *in vivo* on the cytochrome P450 isoforms, and the only results obtained in this way are from research (7). That truth, I did not mean activity directly or concentration of cytochrome P450 isoforms. It was observed that the amount of acetaminophen, which is created in process of O-deethylation of phenacetin catalyzed by cytochrome CYP 1A2, declines.

As comparative vehiculum was used 1% aqueous solution of sodium carboxymethylcellulose (CMC). Chemically it is a semisynthetic cellulose derivative obtained by joining to one of the hydroxyl groups of glucopyranose - carboxymethyl group (-CH_2-COOH).

CMC is used in food science as a viscosity modifier or thickener, and to stabilize emulsions in various products including ice cream. As a food additive, it has number E466. It is also a constituent of many non-food products, such as K-Y Jelly, toothpaste, laxatives, diet pills, water-based paints, detergents, textile sizing and various paper products. It is used primarily because it has high viscosity, is nontoxic, and is hypoallergenic. In laundry detergents it is used as a soil suspension polymer designed to deposit onto cotton and other cellulosic fabrics creating a negatively charged barrier to soils in the wash solution. Carboxymethylcellulose is used as a lubricant in non-volatile eye drops (artificial tears).

Clausen, in study (8), observed that this mucoadhesive polymer – sodium carboxymethylcellulose – displays a permeation enhancing effect. Enhanced permeation was correlated with significant decrease in transepithelial electrical resistance, suggesting that the mechanism of enhacement involves the opening of the tight junctions. 1 % of carboxymethylcellulose sodium salt causes about 10% reduction of the transepithelial electrical resistance of small mucosa as compared to started value and permeation in its 1% aqueous solution increases.

A metabolic probe phenacetin has been selected to study possible methanol pharmacokinetic interactions. Phenacetin metabolism occurs mainly in the liver. Phenacetin O-deethylation has been widely used as an index for CYP 1A2 activity in humans (9, 10) and rats (11-13). In rat liver microsomes high affinity CYP 1A2 and low affinity CYP 2E1 isoforms catalyze the O-deethylation breakdown of phenacetin. Similarly, in human liver microsomes CYP 1A2 isoform is the only high affinity (Km = 31 μM (9), 28 ± 2 μM (14)) component capable of phenacetin O-deethylation. However, at higher phenacetin concentrations, other CYP isoforms (CYPs: 2A6, 2C9, 2C19, 2D6, 2E1) are also involved in phenacetin O-deethylation (9). In rats receiving phenacetin (125 mg/kg b.w.), 63% of the dose are excreted as acetaminophen (paracetamol) in the free or conjugated form, while 21%

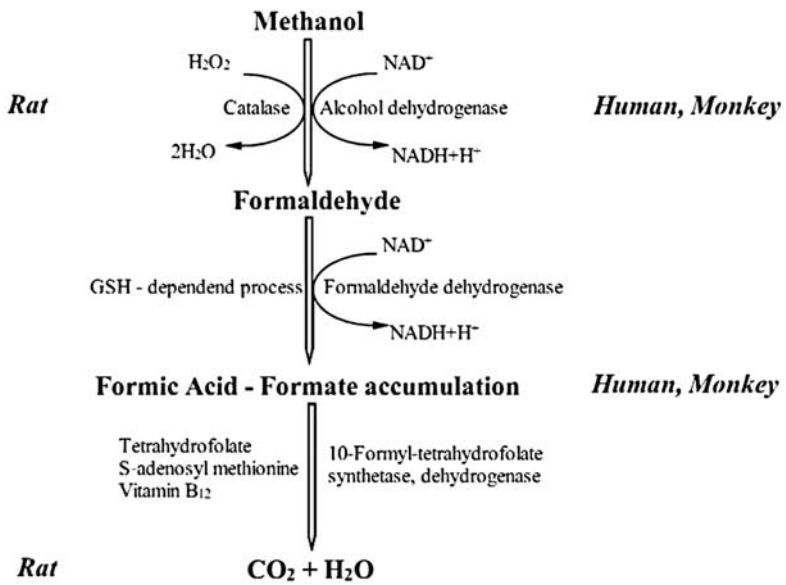

Figure 1. Methanol metabolism (2)

Table 1. Treatment schedule for the first group.

Number of animals	Day of experiment						
	0	14	28	42	56	70	84
6	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}

PHE_{CMC} – phenacetin administered in 1% water solution of carboxymethylcellulose.

Table 2. Treatment schedule for the second group.

Number of animals	Day of experiment								
	0	7	14	21	28	35	42	70	77
6	PHE_{CMC}	PHE_{CMC}	PHE_{Met}	PHE_{Met}	PHE_{Met}	PHE_{CMC}	PHE_{CMC}	PHE_{CMC}	PHE_{Met}

PHE_{CMC} – phenacetin administered in 1% water solution of carboxymethylcellulose. PHE_{Met} – phenacetin administered in 100% methanol.

Table 3. Phenacetin AUC.

$AUC_{0\text{-}4\ h}$ of phenacetin [µg/mL×h] - I group							
Day of experiment	0	14	28	42	56	70	84
K1 = 100% K1 = 37.7 ± 11.3 [µg/mL×h]	100	96.92	114.61	101.35	121.96	108.51	135.10
RSD	30.09	34.09	25.37	29.05	40.49	13.18	21.20
p values							
0	1.0000	0.9031	0.4098	0.9528	0.3736	0.5952	0.1540
14		1.0000	0.4511	0.6611	0.3086	0.4362	0.0422
28			1.0000	0.4915	0.7958	0.6660	0.1837
42				1.0000	0.2692	0.4844	0.0188
56					1.0000	0.4169	0.5973
70						1.0000	0.0896
84							1.0000

$AUC_{0\text{-}4\ h}$ of phenacetin - II group									
Day of experiment	0	7	14	21	28	35	42	70	77
K1 = 100% K1 = 11.67 ± 2.92 [µg/mL×h]	100	96.50	218.82	292.75	250.25	146.14	154.53	160.85	267.80
RSD	24.99	24.41	34.69	33.91	29.05	25.70	18.60	37.80	30.39
p values									
0	1.0000	0.7846	0.0076	0.0017	0.0018	0.0350	0.0026	0.0169	0.0020
7		1.0000	0.0034	0.0046	0.0053	0.0407	0.0137	0.0683	0.0038
14			1.0000	0.1304	0.4147	0.1204	0.0953	0.2064	0.1871
21				1.0000	0.2789	0.0156	0.0171	0.0015	0.5702
28					1.0000	0.0387	0.0140	0.0325	0.5675
35						1.0000	0.6537	0.5442	0.0350
42							1.0000	0.8000	0.0076
70								1.0000	0.0311
77									1.0000

AUC - area under the curve, RSD - relative standard deviation. Each result represents the mean of 6 animals. The results are statistically significant at $p < 0.05$.

of the dose underwent N-deacethylation. Biotransformation pathways of phenacetin in the rat are summarized in Fig. 2 (15, 16).

The aim of this study was to investigate the potential interactions that may occur during the use of methanol as vehiculum and to compare changes when 1% solutions of carboxymethylcellulose were used. The purpose of the investigation was to estimate the effect of methanol on the absorption and the kinetics (AUC) of investigated drug, the biotransformation course, and to identify the events in the long term perspective as well.

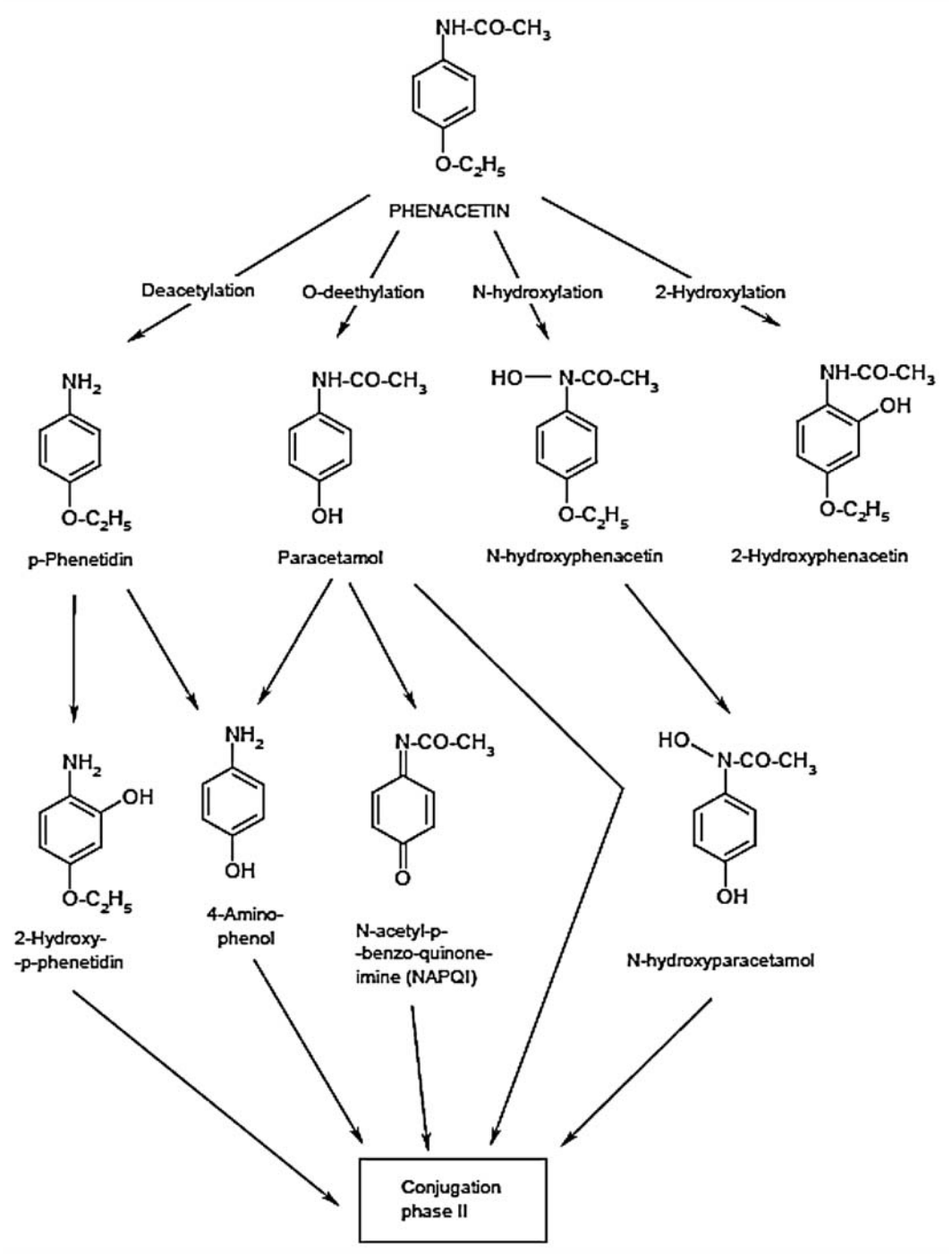

Figure 2. Phenacetin biotransformation in the rat (9, 10)

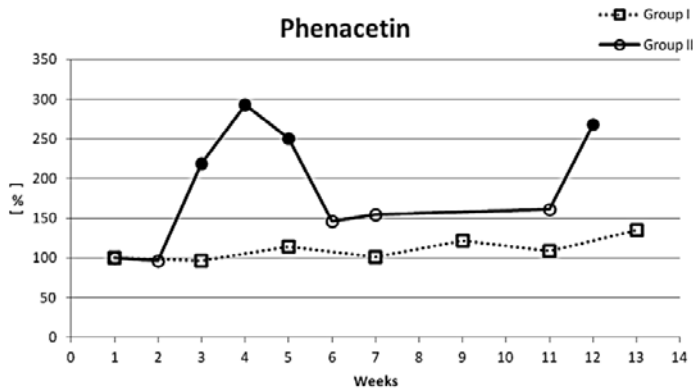

Figure 3. $AUC_{0\text{-}4\,h}$ value for phenacetin in the experiment. Each result represents the mean of 6 animals

Table 4. Acetaminophen AUC.

$AUC_{0\text{-}4\,h}$ of $AUC_{0\text{-}4\,h}$ acetaminophen [µg/mL×h] - I group							
Day of experiment	0	14	28	42	56	70	84
K1 = 100% K1 = 75.67 ± 19.4 [µg/mL×h]	100	78.19	103.14	82.02	86.36	79.95	98.26
RSD	25.62	20.44	30.19	27.78	30.13	16.06	16.90
p values							
0	1.0000	0.2735	0.8494	0.3581	0.4044	0.1328	0.8873
14		1.0000	0.2363	0.6748	0.5923	0.8837	0.1278
28			1.0000	0.2899	0.4198	0.1483	0.7071
42				1.0000	0.7159	0.8288	0.1505
56					1.0000	0.3492	0.3496
70						1.0000	0.0424
84							1.0000

$AUC_{0\text{-}4\,h}$ acetaminophen - II group									
Day of experiment	0	7	14	21	28	35	42	70	77
K1 = 100% K1 = 44.13 ± 5.79 [µg/mL×h]	100	72.93	87.90	73.14	68.70	48.97	60.88	63.53	78.49
RSD	13.13	17.90	23.77	10.56	9.93	7.10	9.85	29.99	24.94
p values									
0	1.0000	0.0005	0.1546	0.0010	0.0032	0.0003	0.0005	0.0099	0.0914
7		1.0000	0.1966	0.9675	0.5714	0.0059	0.0897	0.2251	0.6229
14			1.0000	0.0763	0.0263	0.0082	0.0116	0.1348	0.3493
21				1.0000	0.2253	0.0026	0.0048	0.2952	0.5035
28					1.0000	0.0036	0.0206	0.6084	0.1999
35						1.0000	0.0209	0.1389	0.0241
42							1.0000	0.7450	0.0436
70								1.0000	0.1580
77									1.0000

AUC - area under the curve, RSD - relative standard deviation. Each result represents the mean of 6 animals. The results are statistically significant at $p < 0.05$.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and methanol were purchased from Lab-Scan (Dublin, Ireland), water was obtained from Milli-Q RG ultra pure water system (Millipore, Molsheim, France), and other chemicals including phenacetin and acetaminophen were obtained from Sigma-Aldrich (Steinheim, Germany). All reagents used were of analytical or high performance liquid chromatography (HPLC) grade.

Animals and treatment

Twelve male Wistar cmd(WI)WU rats (initial weight 190 ± 10 g) were kept in plastic cages in animals' cabinet housing (EBECO, LFC 3/16, Germany) with a light/dark cycles of 12/12 h (light on at 6 a.m.). The rats had free access to standard laboratory food (LABOFEED B) and tap water. Food was withdrawn 18 h before phenacetin administration.

The animals were divided into two groups of 6 rats, one of which was the control group and received only phenacetin in carboxymethylcellulose sodium salt, and the other received phenacetin in methanol and CMC. In the control group seven kinetic measurements were performed on animals at two-week intervals, but in second group the intervals were various. The treatment schedule for phenacetin, 1% solution of carboxymethylcellulose and methanol is presented in Tables 1 and 2.

Phenacetin was given intragastrically in a suspension of 1% carboxymethylcellulose water solution or dissolved in methanol at an appropriate dose with simultaneously administered 1 mL of water.

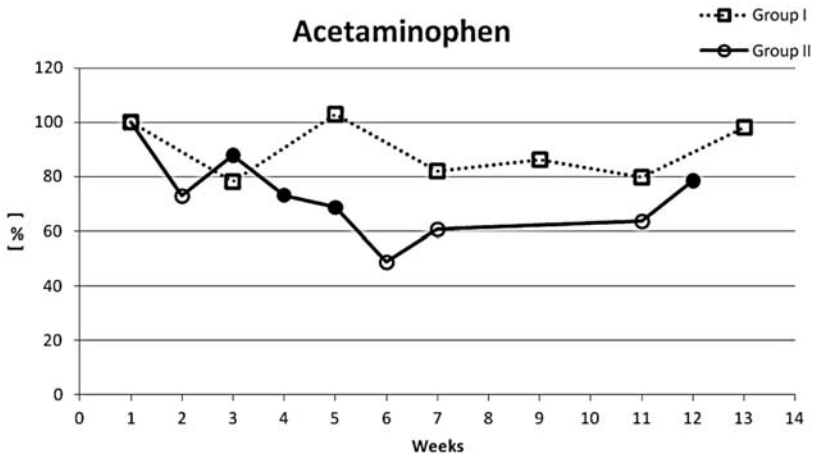

Figure 4. $AUC_{0\text{-}4\,h}$ value for acetaminophen in the experiment. Each result represents the mean of 6 animals.

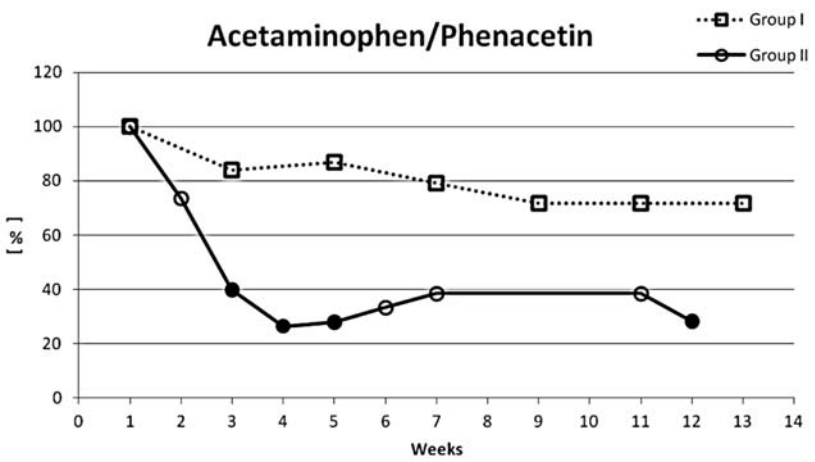

Figure 5. Ratio value of $AUC_{0\text{-}4\,h}$ for acetaminophen to $AUC_{0\text{-}4\,h}$ for phenacetin in the experiment. Each result represents the mean of 6 animals

Table 5. $AUC_{0\text{-}4\ h}$ acetaminophen/ $AUC_{0\text{-}4\ h}$ phenacetin.

$AUC_{0\text{-}4\ h}$ acetaminophen/ $AUC_{0\text{-}4\ h}$ phenacetin [µg/mL×h] - I group							
Day of experiment	0	14	28	42	56	70	84
K1 = 100% K1 = 2.07 ± 0.4 [µg/mL×h]	100	83.91	86.95	79.19	71.81	71.63	71.59
RSD	19.52	33.13	9.39	7.10	16.34	10.33	11.51
p values							
0	1.0000	0.3129	0.1889	0.0490	0.0231	0.0134	0.0222
14		1.0000	0.7248	0.5958	0.1532	0.2834	0.2299
28			1.0000	0.0719	0.0153	0.0014	0.0011
42				1.0000	0.0791	0.1530	0.1136
56					1.0000	0.9771	0.9672
70						1.0000	0.9917
84							1.0000

$AUC_{0\text{-}4\ h}$ acetaminophen/ $AUC_{0\text{-}4\ h}$ phenacetin - II group									
Day of experiment	0	7	14	21	28	35	42	70	77
K1 = 100% K1 = 3.98 ± 1.10 [µg/mL×h]	100	73.60	39.77	26.46	27.98	33.53	38.41	38.51	28.29
RSD	27.77	19.89	22.42	39.67	30.70	24.75	19.53	13.62	6.81
p values									
0	1.0000	0.0815	0.0008	0.0002	0.0003	0.0014	0.0013	0.0009	0.0009
7		1.0000	0.0018	0.0014	0.0010	0.0023	0.0021	0.0015	0.0003
14			1.0000	0.0010	0.0082	0.2052	0.7759	0.4946	0.0174
21				1.0000	0.5496	0.2128	0.0701	0.0048	0.6912
28					1.0000	0.3282	0.0520	0.0038	0.9328
35						1.0000	0.1868	0.1970	0.2231
42							1.0000	0.9745	0.0198
70								1.0000	0.0063
77									1.0000

AUC - area under the curve, RSD - relative standard deviation. Each result represents the mean of 6 animals. The results are statistically significant at $p < 0.05$.

The amount of methanol did not exceed 1 mL/kg b. w. that represented 6.18% of LD_{50}, and did not show any signs of rat intoxication. Each rat received phenacetin at a dose of 100 mg/kg b. w. that represented approximately 6.1% of LD_{50} (17).

Each time phenacetin was administered at the same hour of the day to avoid circadian influences. For the whole experimental period the animals were in good condition and gained weight.

Blood samples of approximately 0,15 mL were collected from the tail vein 0.5, 1, 2, 3 and 4 h after phenacetin ingestion. Between sample collections, animals had free access to water. Serum samples obtained after twofold centrifugation were stored at –20°C until analysis.

The research complied with the Polish binding law and was approved by the Local Ethics Committee for Animal Experiments in Warsaw.

Extraction procedure

Serum (20 µL) was incubated at 37°C with 20 µL of β-glucuronidase/sulfatase (from *Helix pomatia*, type H1, 449 000 units/g, Sigma, 1.4 U of β-glucuronidase/µL).

After 24 h, 20 µL of 8-chlorotheophylline methanolic solution (15 µg/mL) as an internal standard and 440 µL of 0.9% sodium chloride were added to the serum. Probes were subjected to solid phase extraction (SPE). SPE columns used were 1 mL Oasis® HLB disposable extraction columns

packed with 10 mg reversed-phase hydrophilic-lipophilic-balanced (Waters, Ireland). The columns were conditioned (1 mL methanol, 1 mL water) before sample was loaded), washed with 1 mL water and eluted with 1 mL acetonitryle. After elution, the probes were evaporated to dryness at 40°C.

The residue was dissolved in 200 µL of mobile phase and 30 µL aliquots were injected onto HPLC column.

Analytical method

The HPLC system consisted of a Shimadzu LC-10AT solvent delivery module, thermostated CT-10A column oven, SPD-10A UV-VIS detector and autosampler SIL 20AHT. Data processing was performed with a Chroma computer integrator and calculations were done with the aid of Chromix programme.

The assay conditions for phenacetin and acetaminophen were as follows: ABZ+Plus column 5 µm, 15 cm × 4.6 mm i.d. from Supelco with the same precolumn; mobile phase acetonitrile/methanol/0.05 M KH_2PO_4 (135 : 65 : 800, by vol.); flow rate, 1.5 mL/min; temp, 40°C; detection, UV λ = 264 nm.

Calibration curves for phenacetin and its metabolite acetaminophen were established. A linear relationship was observed for the selected concentration ranges of 0.5 to 15 µg/mL. The extraction recovery was 99.8% for phenacetin; 96.7% for acetaminophen and 71.3% for 8-chlorotheophylline.

Statistical analysis

Differences between means were analyzed using t-test for dependent samples with Statistica for Windows (Stat-Soft, Inc.). For statistical comparison all experimental variants performed on a single animal were included. Values for area under the concentration–time curve (AUC) were calculated by the trapezoidal rule to the last point.

RESULTS

After each application of a phenacetin dose of 100 mg/kg b. w. to rat, $AUC_{0\text{-}4\,h}$ of serum phenacetin (Table 3) and acetaminophen (Table 4) concentrations, and the ratio of $AUC_{0\text{-}4h}$ acetaminophen/$AUC_{0\text{-}4\,h}$ phenacetin were estimated (Table 5).

The tables show the value changes of $AUC_{0\text{-}4h}$ for phenacetin, acetaminophen and the ratio of $AUC_{0\text{-}4\,h}$ acetaminophen / $AUC_{0\text{-}4\,h}$ phenacetin in the days of the experiment, expressed as a percentage of the value of the control measurement in 0 day experiment.

DISCUSSION AND CONCLUSION

The purpose of the control group in an experiment is to demonstrate the effects of applied vehiculum. Application of the control group does not exhaust all the possibilities of interaction between vehiculum and the considered drug. These interactions modify the bioavailability of medicinal substances, and thus affect on their pharmacodynamic effects.

The presented results undoubtedly indicate that methanol used as vehiculum significantly alters the bioavailability of phenacetin. As one can see on Figure 3 and in Table 3, dissolving and administration of phenacetin in methanol resulted in 2-3 fold increase in phenacetin $AUC_{0\text{-}4\,h}$ values. Formation of acetaminophen *in vivo* (Table 4 and Fig. 4) was inhibited by methanol, and the ratio of $AUC_{0\text{-}4\,h}$ acetaminophen to $AUC_{0\text{-}4\,h}$ phenacetin has been stabilized at the level of 40% of the start value (Fig. 5).

This observation is supported by *in vitro* studies of Hickman et al. (3) who have tested several organic solvents for inhibitory activity of the *in vitro* microsomal drug metabolism. It was found that methanol at a final concentration of 1% (v/v) decreased by 90% the relative activity of CYP1A2 in the caffeine N3-demethylation.

To compare *in vitro* conditions of that experiment with *in vivo* administration of phenacetin in our previous study, it can be assumed that maximal amount of methanol ingested was approximaly 1 mg/kg b.w. As the amount of blood constitutes 1/20 of the rat body mass (18), the maximum blood methanol concentration can be approximated to 1.6%. This in turn suggests that the amount of methanol present in vehiculum could indeed inhibit in vivo CYP1A2 activity (see Table 4).

In the case of administration of phenacetin in 1% carboxymethylcellulose sodium salt, statistically significant decline in the ratio of $AUC_{0\text{-}4\,h}$ acetaminophen to $AUC_{0\text{-}4h}$ phenacetin value (Table 5) rather is due to the increased amount of phenacetin than decreasing the quantity of paracetamol (first group of the experiment), (Figs. 3 and 4). This may be associated with the ability to enhance permeation (8).

Whether it is the effect of ageing or the effect of methanol cannot be resolved at the present stage. Cytochrome P-450 content increased to 1-month of life, but decreased to 4-month. In the oldest rats (28 month) cytochrome P-450 increased again (19). In the age of two-five months there are no significant changes. Changes in the activity of the transporters with age were observed by other researchers, although it is still a matter of discussion. One of the

most efficient factors controlling absorption of xenobiotics is the activity of protein transporter present in the membrane of any living cell. P-glycoprotein (P-gp) present in the enterocyte brush border exhibits secretory functions limiting the absorption of many drugs. In earlier experiments (20) we have tried unsuccessfully to show the effect of the rat pretreatment with quinidine on phenacetin absorption. In that study design, phenacetin was dissolved in methanol. Because present experiments indicate strong effect of methanol on the phenacetin absorption process, the effect of quinidine should be reinvestigated.

Another explanation for the increased absorption of phenacetin may be age-related changes in transporters activity (21). In rat lymphocytes, an age-dependent increase in P-gp expression and function was observed. An increase in P-gp expression was also evident in the liver, while its reduction was observed in the kidney. In intestinal and endothelial cells of the blood-brain barrier, there was no apparent age-related change, but P-gp expression displayed the greatest variability (22).

The change in the phenacetin kinetics manifests "increasing" kinetic curves and it persists even after stopping administration of phenacetin in methanol, and during the administration of it in 1 % of carboxymethylcellulose sodium salt.

In summary, methanol increases blood phenacetin concentrations and decreases blood acetaminophen concentrations. Relations $AUC_{0\text{-}4\ h}$ for phenacetin metabolite to phenacetin $AUC_{0\text{-}4\ h}$ substrate decrease after administration of methanol and persist at lower levels after its discontinuation. These changes occurred immediately after methanol exposure and the presence of methanol affects on the shape of phenacetin kinetic curves causing them to flatten and to relatively high concentrations in the blood for 4 hours after administration. The age of the animals plays an important role in the metabolism of phenacetin in the rat. The use of methanol as a vehiculum in some cases (e.g., *in vitro* studies, some species of animals) is beneficial in the analysis and interpretation of results.

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CYTOTOXIC AND GENOTOXIC STUDIES OF QUERCETIN, QUERCETIN SODIUM SALT AND QUERCETIN COMPLEXES WITH NICKEL (II) AND ZINC (II)

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Abstract: The aim of this study was to compare the cyto- and genotoxic effects of quercetin, quercetin sodium salt as well as its complexes with nickel (II) and zinc (II) with the use of *Escherichia coli* K-12 *recA::gfp* microbial biosensor strain containing transcriptional fusion between DNA-damage genotoxin-inducible recA promoter involved in the SOS regulon response and fast folding GFP (green fluorescent protein) variant reporter gene – *gfpmut2*. Obtained results indicate that *recA::gfpmut2* genetic system was a sensitive biosensor to the most of tested chemicals. The complex of quercetin with sodium, nickel and zinc increased (and in some cases modulated) the reactivity of *recA* promoter in relation to control sample. The results indicated that *E. coli* K-12 *recA::gfp mut2* strain could be potentially useful for monitoring of cytotoxic and genotoxic effect of some biological natural compounds, potentially used in anticancer chemoprevention and therapy.

Keywords: anticancer, quercetin, metal complexes, *recA* promoter

Chemoprevention is defined as the use of natural, synthetic or biological agents to reverse, suppress or prevent either the initial phases of carcinogenesis or the progression of premalignant cells to invasive disease. The main sources of potentially non-toxic chemo-preventive molecules are fruits and vegetables. These molecules can exert a cancer-preventive effect and therefore are termed as "chemo-preventers". The most studied natural compounds are: curcumin, quercetin (Q), resveratrol, luteolin, genistein, lycopene and, in general, flavonoids and polyphenols. In laboratory studies were used aqueous extracts from plants and showed antioxidant, antimicrobial, anti-proliferative, pro- or anti-apoptotic properties of the aforementioned molecules (1-5).

Quercetin (3,5,7,3´,4´-pentahydroxyflavone) belongs to the group of dietary bioflavonoids. Q is abundant in variety of foods including apples, berries, brassicaceous vegetables, grapes, onions, shallots, tomatoes and tea as well as many seeds, nuts, barks and leaves. The chemical structure of Q consists two aromatic rings A and B, linked by an oxygen containing heterocyclic ring C (Fig. 1) (6-8).

Different studies demonstrated that Q is one of the most potent anti-oxidants with the ability to

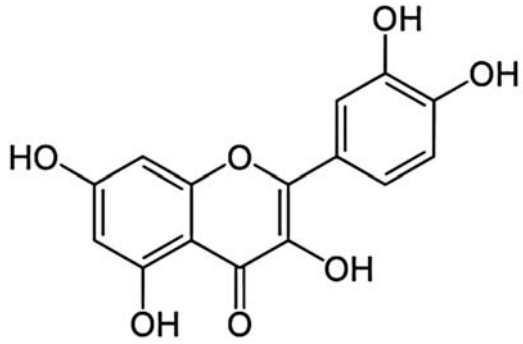

Figure. 1. Chemical structure of quercetin

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inhibit oxidative species generating enzymes such as xanthine oxidase, LOX (lysyl oxidase), and nicotinamide adenine dinucleotide phosphate oxidase. Because of its potent antioxidant and metal ion chelating capacity, Q has been reported to be effective in inflammation, arteriosclerosis, bleeding, allergy and swellings. Q is the main flavonoid in diet with a potential in the treatment of cardiovascular and neurodegenerative diseases. Additionally, laboratory studies has exhibited promising antitumor property of Q in many human cancer cells both *in vitro* and *in vivo*. The main molecular mechanisms of anti-cancer activity of Q are connected with its influence on cell cycle regulation, interaction with type II estrogen binding sites and tyrosine kinase inhibition (2, 4, 6, 9-17).

Glycosides are the most popular form of Q found in the natural products. They are too polar to penetrate the intestinal membranes and hence, are not easily absorbed. Because of that, the bioavailability is much lowered and its anti-tumor effect has been restricted to a great extent. In order to overcome this deficiency, some chemical modifications are done in the number of hydroxyl groups. The presence of methoxy group in the B ring or lipophilicity are known to influence the bioavailability of flavonoids. Q has been combined with different anti-cancer compounds or chemicals to enhance inhibition of various tumors, including prostate cancer. Due to its specific planar chemical structure, Q readily forms chelates with metal ions. Those complexes are reported to have various important biological activities, and most of them exhibit higher antioxidant abilities than the ligand flavonoids (10-17).

During the last decade, it has been shown that bacterial-based genotoxicity reporter systems display an important role in the rapid, first-step biological screening and detection of DNA damaging chemicals, drugs and potential drug candidates. These bacterial biosensors systems consist genetically engineered microorganisms, 'tailored' to generate a quantifiable signal that reflects the genotoxic potency of the tested chemicals or drugs. Such assays possess several significant advantages including rapid response times, high reproducibility, facility of use and low operational cost (18-21, 23, 24).

The *recA* promoter of the RecA recombinase gene, which plays a key role in the SOS response by its co-protease activity on the LexA repressor, has served as the basis of several genotoxicity microbial biosensors. The *recA* promoter in transcriptional fusion with reporter genes, such as green fluorescent protein (*gfp*) or luciferase (*lux, luc*) in microbial biosensors were tested and indicated genotoxic sensitivity against 4-nitroquinoline-N-oxide (4-NQO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), mitomycin C (MMC) and some others different anticancer drugs, UV radiation, hydrogen peroxide, formaldehyde, cumene hydroperoxide, streptonigrin and other chemicals (23, 24). In our earlier works we studied the influence of selected metals on the molecular structure and electronic distribution of biologically important ligands (e.g., benzoic, salicylic, nicotinic acids and selected phenolic acids). Composition of complexes and molecular structure was investigated by FT-IR, FT-Raman, ^{1}H, ^{13}C NMR, XRD, thermogravimetry and quantum chemical calculations. Biological activity (microbiological and anticancer properties) we investigated in cooperation with Medical University in Bialystok, National Drug Institute and MD Anderson Cancer Centre, (Houston, USA) (25, 26). Transition metals having high ionic potential (such as Fe (III), Cr (III), Ln (III), Y (III), Zn (II)) and Al (III) stabilize the electronic system of ligands, whereas alkali metals and heavy toxic metals, such as Ag (I), Hg (I) and (II), Cd (II), Pb (II) disturb the electronic system of ligands. Moreover, in many cases we showed the correlation between the molecular structure and microbiological activity of investigated ligands (27-31). Our present work is a part of a broad subject concerning studies on improvement of selectivity and activity of the chosen drugs and natural compounds (phenolic acids and flavonoids) characterized with anticancer properties, during the complexation by metals. Particularly, the aim of this study was to compare the cyto- and genotoxic effects of the Q, Q sodium salt and Q sodium salt/transition metals complexes (Zn (II) and Ni (II)) with use of *Escherichia coli* K-12 *recA::gfp* microbial biosensor. Cytotoxic properties of tested chemicals were measured on the basis on optical density (OD) values. Genotoxic properties were indicated on the basis on the specific fluorescence intensity (SFI) values.

MATERIALS AND METHODS

Synthesis

Zn (II) and Ni (II) complexes with Q were synthesized by the following method. To the 0.01 mol of Q dissolved in MeOH (20 mL) 0.02 mol of $ZnCl_2$ or $NiCl_2$ was added and NaOH solution (C = 0.1 mol/L) to the pH = 10. The sodium salt of Q was synthesized, namely 0.01 mol of Q was dissolved in MeOH (20 mL) and then the 0.02 mol of NaOH was

added. Then, the obtained solutions were stirred at room temperature for 1.5 h. After stirring, the reaction mixtures were filtered, and the filtrates were evaporated slowly at room temperature. The resulting dark yellow products were washed with *t*-butanol and dried in a desiccator. The composition of studied compounds was revealed on the basis of elemental and thermogravimetric studies. The results of elemental analysis were as follows: $Na(C_{15}H_9O_7)\cdot 1H_2O$ $\%C_{exp} = 51.46$ ($\%C_{calc} = 52.59$), $\%H_{exp} = 3.07$ ($\%H_{calc} = 3.21$); $Ni(C_{15}H_8O_7)\cdot 2.5H_2O$ $\%C_{exp} = 42.81$ ($\%C_{calc} = 44.56$), $\%H_{exp} = 3.03$ ($\%H_{calc} = 3.22$); $Zn(C_{15}H_8O_7)\cdot 4H_2O$ $\%C_{exp} = 39.92$ ($\%C_{calc} = 41.13$), $\%H_{exp} = 3.66$ ($\%H_{calc} = 3.66$). All chemicals were purchased from Sigma-Aldrich and used without purification.

Bacteria strain and plasmid

In the experiment *Escherichia coli* K-12 MG1655 stationary phase cells: *Escherichia coli* K-12 *recA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2*, genetically modified were used. They contained a pUA66 plasmid-borne transcriptional fusion between DNA-damage inducible, oxidative stress *recA* promoter involved in the SOS regulon response and fast folding GFP variant reporter gene-*gfpmut2*. The genetic structure of pUA66 plasmid is described in the work of Zaslaver et. al. (22). In the present work, a more stable and fast folding mutant of *gfp* gene – *gfpmut2* with excitation and emission wavelengths of 485 and 507 nm, respectively, was used.

Bacteria growth condition

Escherichia coli K-12 MG1655 strains: *Escherichia coli* K-12 *recA*::*gfpmut2* and *Escherichia coli* K-12 *promoterless*::*gfpmut2* were cultured overnight in LB agar medium (Merck, Germany) at 30°C supplemented with 100 μg/mL of kanamycin (Sigma-Aldrich, Germany). Colonies were carried to LB broth medium (10 g NaCl, 10 g tryptone and 5 g yeast extract per 1000 mL of distilled water) with 100 μg/mL of kanamycin and incubated overnight at 30°C. Afterwards, the cells were washed with PBS (phosphate buffered saline) (1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.2 g KCl, 8 g NaCl in 1000 mL destilled water, pH = 7) buffer.

Monitoring of bacteria growth and concentration

At the beginning of the experiment, the initial bacteria cells density was standardized to OD (optical density) value by using spectrophotometer (Glomax®, Multi Detection System, Promega) at the wavelength of 600 nm. The concentration of bacteria cells per mL of PBS was assessed by series dilutions system and expressed as CFU (colony forming units)/mL values.

Dynamic growth of bacteria strains treated with chemicals was monitored by the use of standard spectrophotometer analysis of optical density values at the wavelength of 600 nm.

The values of bacteria growth inhibition (GI) during the treatment with compounds at the start of bacteria incubation with chemicals (time 0 and after 3 and 24 h) was calculated according to the formula:

$$GI\ (\%) = OD_{CS}\ (\%) - D_{ODTS}\ (\%)$$

where: OD_{CS} (%) = optical density of control sample = 100 %, D_{ODTS} (%) = the decrease in the value of optical density of bacteria samples treated with chemicals in relation to OD value of control sample.

Genotoxicity testing of quercetin and its derivatives

One milliliter of stationary phase bacteria cells (3×10^7 CFU/mL; OD = 0.2) were suspended in 4 mL of PBS buffer and the following chemicals were used for genotoxicity testing: Q, Q sodium salt and Q sodium salt/transition metals complexes (Zn (II) and Ni (II)) at concentrations of: $3310\cdot 10^{-3}$ μmol/L; $3310\cdot 10^{-4}$ μmol/L; $3310\cdot 10^{-5}$ μmol/L; $3310\cdot 10^{-6}$

Table 1. Applied concentrations of tested chemicals.

Chemicals	Concentrations (μmol/L)			
$Na(C_{15}H_9O_7)\cdot 1H_2O$ Quercetin sodium salt	$2930\cdot 10^{-3}$	$2930\cdot 10^{-4}$	$2930\cdot 10^{-5}$	$2930\cdot 10^{-6}$
$Ni(C_{15}H_8O_7)\cdot 2.5H_2O$ Quercetin complex with nickel	$2480\cdot 10^{-3}$	$2480\cdot 10^{-4}$	$2480\cdot 10^{-5}$	$2480\cdot 10^{-6}$
$Zn(C_{15}H_8O_7)\cdot 4H_2O$ Quercetin complex with zinc	$2290\cdot 10^{-3}$	$2290\cdot 10^{-4}$	$2290\cdot 10^{-5}$	$2290\cdot 10^{-6}$
$C_{15}H_{10}O_7$ Quercetin	$3310\cdot 10^{-3}$	$3310\cdot 10^{-4}$	$3310\cdot 10^{-5}$	$3310\cdot 10^{-6}$

μmol/L for $C_{15}H_{10}O_7$ - Q, $2930 \cdot 10^{-3}$ μmol/L; $2930 \cdot 10^{-4}$ μmol/L; $2930 \cdot 10^{-5}$ μmol/L and $2930 \cdot 10^{-6}$ μmol/L for $Na(C_{15}H_9O_7) \cdot 1H_2O$ - Q sodium salt, $2480 \cdot 10^{-3}$ μmol/L; $2480 \cdot 10^{-4}$ μmol/L; $2480 \cdot 10^{-5}$ μmol/L and $2480 \cdot 10^{-6}$ μmol/L for $Ni(C_{15}H_8O_7) \cdot 2.5H_2O$ – Q complex with nickel and $2290 \cdot 10^{-3}$ μmol/L; $2290 \cdot 10^{-4}$ μmol/L; $2290 \cdot 10^{-5}$ μmol/L and $2290 \cdot 10^{-6}$ μmol/L for $Zn(C_{15}H_8O_7) \cdot 4H_2O$ – Q complex with zinc (Table 1).

The concentration range of the analyzed drugs was selected experimentally from the minimum level of *recA::gfp* construct sensitivity and according to the reviewed references recommendation (6-18). The time of bacteria incubation with drugs (up to 3 h) was estimated for monitoring the sensitivity of *recA::gfp* genetic construct. The control sample - *Escherichia coli* K-12 *recA*::*gfpmut2* strain in PBS buffer was not treated with chemicals. For verification the correct activity of *recA* promoter, *Escherichia coli* K-12 strain containing pUA66 plasmid without the promoter - *Escherichia coli* K-12 *promoterless*::*gfpmut2* - was used as the control one. Additionally, for assessment of genotoxic sentivity of *recA::gfp* construct, 4% acetone was used as the negative control and 50 μM methylnitronitrosoguanidine (MNNG, known genotoxin) as the positive control.

Analytical method for the intensity of *gfp* gene fluorescence (IF) analysis

After exposition of bacteria cultures to tested chemicals, the strains were washed with PBS buffer and the intensity of fluorescence of *gfp* gene in the volume of 1 mL of bacteria cells suspension (2×10^5 CFU/mL) in PBS buffer was measured in the spectrofluorometer (Glomax®, Multi Detection System, Promega). The measurements were done at excitation and emission wavelengths of 485 and 507 nm, respectively.

Assessment of SFI values

The specific fluorescence intensity (SFI) value measured with a spectrofluorometer is defined as culture fluorescence (IF) divided by culture at a cell density of OD at 600 nm. SFI value was calculated according to the below formula for monitoring the dynamic of *gfp* expression after bacteria treatment with chemicals:

$$SFI = \frac{IF}{OD}$$

where: SFI = specific fluorescence intensity, IF = the raw fluorescence intensity of the strains at excitation and emission wavelengths of 485 and 507 nm, OD = optical density at 600 nm of the strains.

Detection of the percentage stimulation of green fluorescence protein expression ($S_{gfpesp.}$) value

For the increased SFI values with the level of *gfp* expression in comparison with the control sample the percentage stimulation of *gfp* ($S_{gfpexp.}$) was calculated according to the formula:

$$S_{gfpexp.}(\%) = I_{TS}(\%) - SFI_{CS}(\%)$$

where I_{TS} (%) = the increase for SFI values for tested compounds sample in comparison with the control sample, SFI_{CS} (%) = SFI for the control sample = 100%.

Assessment of F_I values

For each concentration of tested chemicals the induction factors (F_I) were calculated. $F_I = (Fl_I/OD_0)/(Fl_0/OD_I)$, where Fl_I is the raw fluorescence of the culture treated with DNA - damaging compound; Fl_0 is the raw fluorescence of the control sample without genotoxin; OD_I is the optical density at 600 nm of treated culture and OD_0 is the optical density of the control sample.

The SFI, $S_{gfpexp.}$ and F_I values express the potency of influence of tested chemicals on the sensitivity of *recA::gfp* construct.

Classification of tested drugs as genotoxins

The F_I values were calculated for classification of tested drugs as genotoxins. According to Ptitsyn et. al. (24) and Kostrzyńska et. al. (25), the chemical was identified as a genotoxin, when its induction factor was higher than 2 ($F_I = 2$).

Statistical analysis

Statistical data obtained from eight measurements are expressed as a mean ± standard deviation (SD) (n = 8). The data were analyzed by the use of standard statistical analyses, including one-way Student`s test for multiple comparisons to determine the significance between different groups. The values for $p \leq 0.05$ were considered significant.

RESULTS

Escherichia coli K-12 MG1655 *recA*::*gfpmut2* strain 3 h treatment with quercetin (Q), quercetin sodium salt (Q-Na), zinc complex of quercetin (Q-Zn) and with Ni (II) (Q-Ni) showed significant increase ($p \leq 0.05$) in SFI and $S_{gfpexp.}$ values compared to non-treated cells for higher concentrations ($2930 \cdot 10^{-3}$ - $2930 \cdot 10^{-5}$ μmol/L for $Na(C_{15}H_9O_7) \cdot 1H_2O$; $2480 \cdot 10^{-3}$ - $2480 \cdot 10^{-5}$ μmol/L for $Ni(C_{15}H_8O_7) \cdot 2.5H_2O$; $2290 \cdot 10^{-3}$ - $2290 \cdot 10^{-5}$ μmol/L for $Zn(C_{15}H_8O_7) \cdot 4H_2O$ and $3310 \cdot 10^{-3}$ - $3310 \cdot 10^{-5}$ μmol/L for $C_{15}H_{10}O_7$) of tested compounds (Table 2). In the case of the low-

est concentration of Q-Ni ($2480 \cdot 10^{-6}$ µmol/L) also a significant increase in SFI and S_{gfpexp} values was observed.

Figure 2 shows the dynamics of the SFI values for *E. coli* K-12 *recA::gfp mut2* treated with four different concentration of Q, Q-Na, Q-Zn and Q-Ni after 3 h incubation compared to the control sample (not-treated with these substances).

The monitoring of bacteria cultures growth (OD) at the start of bacteria treatment (time 0) and after 3 h of incubation with chemicals indicated a significant increase in GI (growth inhibition) values for quercetin and Q-Ni for 3 h treatment (Fig. 3). Q-Ni strongly inhibited the GI value comparable to Q.

Bacteria incubation with PBS buffer (the control sample) without any compounds addition resulted in no statistically differences in OD value from 0 to 3 h continuous cultivation.

Bacteria treatment with Q resulted in a progressive significant stimulation of SFI values for $3310 \cdot 10^{-3}$ - $3310 \cdot 10^{-5}$ µmol/L for 3 h incubation compared to the control sample. The maximum point for SFI value (S_{gfpexp}.= 60.5%) was for Q in the concentration of $3310 \cdot 10^{-3}$ µmol/L and 3 h of incubation time.

Bacteria cells administrated with Q-Na, exerted some influence on SFI and the parameters with the maximum point for SFI (S_{gfpexp} = 75.53%) were for $2930 \cdot 10^{-4}$ µmol/L of Q-Na after 3 h incubation. At concentration of $2930 \cdot 10^{-6}$ µmol/L no significant differences in SFI values between Q and Q-Na were observed.

Treatment of bacteria cells with Q-Zn significantly stimulated the SFI values, especially at $2290 \cdot 10^{-4}$ and $2290 \cdot 10^{-5}$ µmol/L concentrations (94.6 and 82.34%, respectively). For bacteria incubation

Table. 2. SFI values for stationary phase *E. coli* K-12 *recA::gfp mut2* treated with quercetin (Q), quercetin sodium salt (Q-Na), zinc complex of quercetin (Q-Zn) and nickel complex of quercetin (Q-Ni)) in comparison with the control sample (bacteria strain in PBS buffer), F_I – induction factor values, $S_{gfpexp.}$(%) – the percent of stimulation of *gfp* expression after treatment of bacteria cells with tested chemicals in comparison with the control sample (100 %).

Tested sample	Concentration (C) (µmol/L)			
	10^{-3}	**10^{-4}**	**10^{-5}**	**10^{-6}**
Control sample SFI ± SD	111.81 ± 6.42	111.81 ± 6.42	111.81 ± 6.42	111.81 ± 6.42
Quercetin (Q) C (µmol/L): $3310 \cdot 10^{-3}$ - $3310 \cdot 10^{-6}$ SFI ± SD	175 ± 7.82^{a}	172.1 ± 6.37^{a}	161.57 ± 7.58^{a}	118.11 ± 4.93
$S_{gfpexp.}$(%)	60.5	53.92	44.50	5.63
Quercetin sodium salt C (µmol/L): $2930 \cdot 10^{-3}$ - $2930 \cdot 10^{-6}$ (Q-Na), SFI ± SD	169.41 ± 6.0^{a}	196.26 ± 16.90^{a}	163.76 ± 6.80^{a}	111.04 ± 15.30
$S_{gfpexp.}$(%)	51.52	75.53	46.46	-
Zinc complex of quercetin (Q-Zn) C (µmol/L): $2290 \cdot 10^{-3}$ - $2290 \cdot 10^{-6}$ SFI ± SD	185.95 ± 9.62^{a}	217.5 ± 13.50^{a}	203.88 ± 8.00^{a}	118.11 ± 7.60
$S_{gfpexp.}$(%)	66.31	94.60	82.34	5.63
Nickel complex of quercetin (Q-Ni) C (µmol/L):$2480 \cdot 10^{-3}$ - $2480 \cdot 10^{-6}$ SFI ± SD	297.5 ± 9.13^{abcd}	475 ± 12.93^{abcd}	259.16 ± 10.07^{abc}	135.24 ± 6.52^{a}
$S_{gfpexp.}$(%)	166.07	324.83	131.78	20.95
F_I	2.66	3.24	2.32	-

Data points represent mean values ± SD; n = 8; a - significantly different from control group ($p < 0.05$); b - significantly different from quercetin (Q) group ($p < 0.05$); c - significantly different from quercetin sodium salt (Q-Na) group ($p < 0.05$); d - significantly different from zinc complex of quercetin (Q-Zn) group ($p < 0.05$).

with Q-Ni the F_I values were below 2 at the concentration of $2480 \cdot 10^{-3}$ - $2480 \cdot 10^{-5}$ µmol/L. For these concentration bacteria cells treatment with Q-Ni resulted in a progressive increase in SFI values to 166.07; 324.83 and 131.78% of *gfp* expression stimulation in comparison to the control.

To compare the level of activity of tested chemical to *gfp* expression in *E. coli recA::gfp* the strongest influence was noticed for application of Q-Ni.

In this experiment, for assessment of genotoxic sensitivity of a *recA::gfp* genetic biosensing system, 4 % acetone was tested as the negative control. In the case of this chemical there was no increase in F_I values for 3 h and 24 h of incubation. Methylnitronitrosoguanidine (MNNG) – known genotoxin at the concentration of 50 µM - was used as the positive control. For this analyte $F_I = 8.4$ for 24 h incubation time and $F_I = 2.8$ for 3 h. These results proved stronger

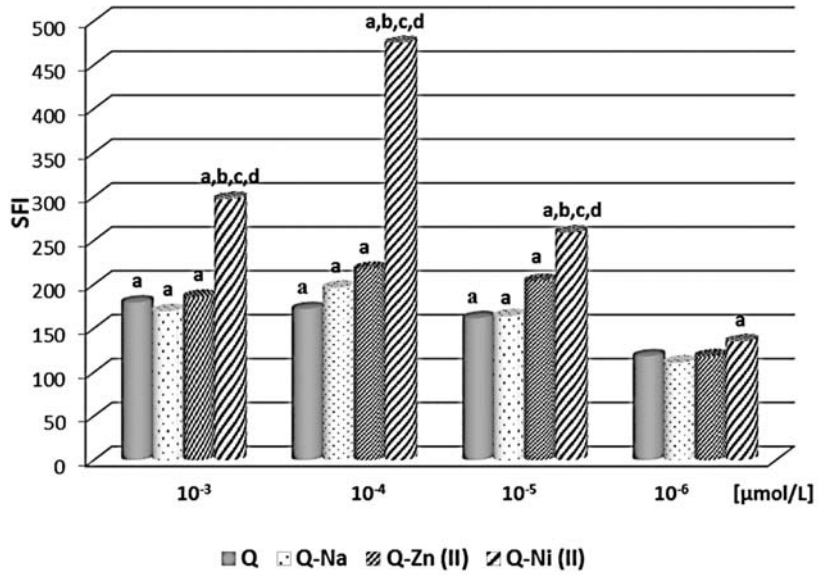

Figure 2. The dynamics of the SFI values for *E. coli* K-12 *recA::gfp mut2* treated with quercetin (Q), quercetin sodium salt (Q-Na), zinc complex of quercetin (Q-Zn) and nickel complex of quercetin (Q-Ni)). SFI values are expressed as the percent of *gfp* gene IF stimulation of bacteria cells normalized by dividing by optical density value. Data points represent mean values ± SD; n = 8; a - significantly different from control group ($p < 0.05$); b - significantly different from quercetin (Q) group ($p < 0.05$); c - significantly different from quercetin sodium salt (Q-Na) group ($p < 0.05$); d - significantly different from zinc complex of quercetin (Q-Zn) group ($p < 0.05$)

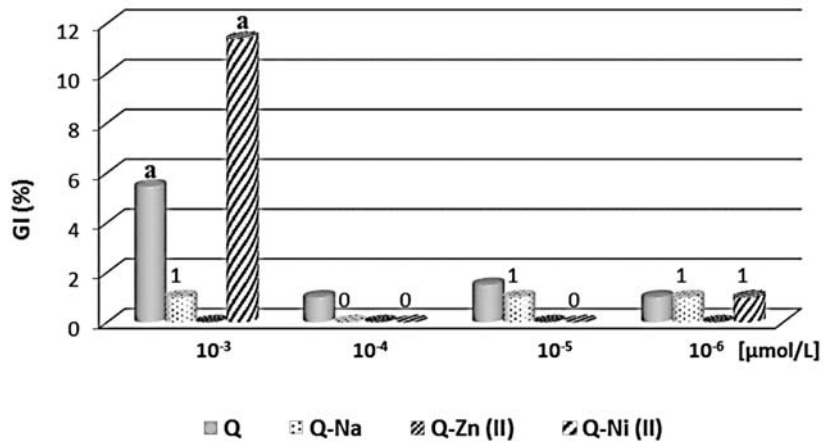

Figure 3. The comparison of GI values of *E. coli* K-12 *recA::gfp* cells after 3 h of incubation with quercetin (Q), quercetin sodium salt (Q-Na), zinc complex of quercetin (Q-Zn) and nickel complex of quercetin (Q-Ni). Data points represent mean values ± SD; n = 8; a - significantly different from control group ($p < 0.05$)

sensitivity of a *recA::gfp* biosensing system for MNNG than for an acetone stressor.

DISCUSSION

Q is a bioflavonoid found in a wide range of fruits, vegetables and beverages. Among those, onion, apple and red wine are good sources of quercetin. Q possesses a wide spectrum of pharmacological properties including antioxidant anti-inflammatory aspects. This natural compound possesses cancer-preventive and anticancer effects on various types of cancer cells (1-5).

Results obtained in this study indicate that treatment of bacteria cells with Q, Q-Na, Q-Zn and Q-Ni lead to over 2-fold stimulation (F_I = 2.7 and 2.32 in the case of Q-Ni of bacteria genotoxin-sensitive *recA* promoter and *gfp* gene expression. This indicates that complexation of Q with sodium, zinc and nickel modulate and increase the reactivity of *recA* promoter in relation to quercetin in *E. coli* K-12 *recA::gfp mut2* living bacteria cells.

Our experimental data confirmed the sensitivity of *recA* promoter to Q and its complexes with sodium and selected transition metals. *RecA* promoter is one of the best known promoter from SOS bacteria regulon with well proved genotoxic sensitivity to anticancer drugs, drugs candidates and different chemicals with pharmacological potency (18, 19, 21-24).

The molecular mechanisms of anticancer activity of Q is mainly connected with induction of growth arrest in G1 or G2 of mitotic phase, apoptosis and inhibition of angiogenesis as evidenced by down-regulating the expression of oncogenes (HER2, H-ras, K-ras, c-myc or COX-2) and mutant p53 or up-regulating cell cycle control proteins (p21WAF1 and p27KIP1) (25).

The results of experiment provided the conformation of the possible influence of Q on the genes expression, similarly to some authors mentioned above (10-14, 25). In almost 80% of cases, there were significant differences (in comparison to the control sample) in the level of *recA* promoter sensitivity and *gfp* expression after bacteria treatment for 3 h with all applied concentrations of Q and its complexes with sodium and transition metals.

The specific chemical structure of Q provides the possibility to form chelates with metal ions. Those complexes are reported to have various important biological activities (9-22).

The results of above experiment showed the difference of the cyto- and genotoxic potency of Q complexes with zinc and nickel and sodium salt of Q, in comparison to the Q group. Q exposure (up to 3 h) resulted in a progressive stimulation of *recA* promoter activity and *gfp* gene expression. The strongest stimulation of *gfp* expression by Q was noticed when $3310 \cdot 10^{-3}$ and $3310 \cdot 10^{-4}$ µmol/L of chemical was added rather than in the case of lower concentrations. Zinc and nickel complexes of Q enhanced the sensitivity of *recA* promoter and stimulated the *gfp* gene expression. The two concentrations ($2290 \cdot 10^{-4}$; $2290 \cdot 10^{-5}$ µmol/L and $2480 \cdot 10^{-4}$; $2480 \cdot 10^{-5}$ µmol/L for) of zinc and nickel complexes of Q caused the strongest intensification of SFI values as compared to Q-Na, Q and control samples. The highest level of reactivity to *recA* promoter was noticed for Q-Ni at concentration of $2480 \cdot 10^{-4}$ and $2480 \cdot 10^{-5}$ µmol/L with F_I value above 2. It suggests the possible direct or indirect influence of Q metals complexes on transcription of *recA::gfp* genetic construct in *E. coli*.

Our results showed significant inhibition of bacteria cells growth treated with Q and Q-Ni. Administration of Q importantly intensified cytotoxic effect on living bacteria cells after 3 h incubation, as compared to Q-Ni. Our results are in agreement with earlier studies of some other authors, who demonstrated, that Q inhibits the proliferation and induces apoptosis of cancer cells (6-8). In addition, Q inhibits the activities of several tyrosine and serine–threonine kinases linked to cell surface receptors, which transduced survival pathways (PI3K/Akt/PKB) (32).

The biological and anticancer activity of Q and its different chemical derivatives and complexes with metals are the main topic of scientific interests in experimental studies on bacteria, human cells and other organisms.

CONCLUSIONS

1. Q-Na and Q complexes with zinc and nickel modulate and increase the reactivity of *recA* promoter in relation to quercetin as compared to control sample.

2. Q-Ni got the strongest stimulation of *recA::gfp* genetic systems in comparison to the rest of the tested chemicals and control sample.

3. The results indicated that *E. coli* K-12 *recA::gfp mut2* strain could be potentially useful for monitoring of cyto- and genotoxic effect of natural compounds potentially useful in anticancer chemoprevention and therapy.

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DRUG SYNTHESIS

DESIGN, SYNTHESIS AND ANTICANCER ACTIVITY OF SOME NOVEL 1,2,4-TRIAZOLES CARRYING BIOLOGICALLY ACTIVE SULFONAMIDE MOIETIES

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Abstract: Thirteen novel 1, 2, 4-triazoles incorporating a biologically active sulfonamide moieties **1**-**13** were designed and synthesized. The structures of the prepared compounds were elucidated on the basis of elemental analyses, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. All the newly synthesized compounds were evaluated for their *in vitro* anticancer activity against various cancer cell lines. The corresponding triazole carrying a biologically active free sulfonamide with unsubstituted phenyl ring **1** and triazole bearing sulfonamide with dimethylpyrimidine **11** were the most potent in this study which showed higher activity than the reference drug 2',7'-dichlorofluorescein (DCF). Cytotoxic screening of the tested compounds could offer an encouraging framework in the field that may lead to the discovery of potent anticancer activity.

Keywords*:* Synthesis, triazoles, benzenesulfonamides, anticancer activity

From the literature survey it was found that 1,2,4-triazoles and their derivatives have great importance in medicinal chemistry and can be used for the synthesis of numerous heterocyclic compounds with different biological activities such as antiviral, antibacterial, antifungal, antituberculosis, anticonvulsant, antidepressant, anti-inflammatory and anticancer activities (1). They have been reported to be inhibitors of glycogen synthase kinase-3 (2), antagonists of GABA receptors (3, 4), agonists of muscarine receptors (5), be neuroleptic (6), and these compounds also show anti-HIV-1 (7), cytotoxic (8), antihistaminic (9), and antiproliferative (10) activities. The advent of high-throughput screening systems has allowed us to evaluate a large number of small molecules in parallel and automated fashions. In response to this screening innovation, one of the greatest concerns in recent drug discovery programs has been directed toward how to design and prepare compound libraries for getting "hits" in various biological assays (11). In this regard, historical reviews of drug discovery often give us practical lessons. One highly informative example is represented by the sequential development of sulfonamide therapeutics such as antibiotic sulfa drugs, insulin-releasing hypoglycemic agents, carbonic anhydrase inhibitory diuretics, high-ceiling diuretics, and antihypertensive drugs (12-14). These diverse pharmacological effects were serendipitously found through the serial derivatization of a single chemical structure of sulfanilamide, indicating that the sulfonamide moiety is a crucial functionality capable of interacting with multiple cellular targets. Therefore, we have seriously considered that novel anticancer chemotherapeutics might be discovered from the sulfonamide class. E7010 was shown to reversibly bind to the colchicine site of β-tubulin, thereby halting mitosis (15-19). The compound exhibited good *in vivo* efficacy against rodent tumors and human tumor xenografts (20). As a *p.o.*-active antimitotic agent, E7010 demonstrated objective responses in 2 of 16 patients in the single-dose study of phase I trials; spinal cord metastasis was reduced by 74% in a patient with uterine sarcoma, and a minor response was observed in a pulmonary adenocarcinoma patient (21). In contrast to E7010,

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E7070 was found to block cell cycle progression of P388 murine leukemia cells in the G_1 phase, accompanied by a decrease in the S-phase fraction (16). Although its precise mode of action has not yet been determined, E7070 appears to be considerably different from conventional anticancer drugs in clinical use with respect to its cell cycle effect and its tumor type selectivity (18-22). Furthermore, preclinical animal tests established the promising efficacy of E7070 against human tumor xenografts (22). Thus, the compound has progressed to clinical evaluation in collaboration with the European Organization for Research and Treatment of Cancer (23). In the phase I setting, one patient with a uterine adenocarcinoma experienced a partial response with a > 50% shrinkage of measurable tumors after *i.v.* administration on the weekly schedule (24). Another partial response was reported in a patient with breast cancer on the daily × 5 schedule (25). Phase II studies of E7070 are currently ongoing in Europe and the United States. On the basis of the significant observations described above, together with our interest in the synthesis of biologically active heterocyclic compounds (26-31) we decided to synthesize several novel sulfonamide carrying a biologically active 1,2,4-triazole analogs of E7010, E7070, ER-67865, and ER-68487 (Fig. 1) to evaluate their anticancer activity.

EXPERIMENTAL

Chemistry

Melting points (uncorrected) were determined in open capillary on a Gallenkamp melting point apparatus (Sanyo Gallenkamp, UK). Precoated silica gel plates (Kieselgel 60 F254, 0.25 mm, Merck, Germany) were used for thin layer chromatography. A developing solvent system of chloroform/methanol (8 : 2, v/v) was used and the spots were detected by ultraviolet light. IR spectra (KBr disc) were recorded using an FT-IR spectrophotometer (Perkin Elmer, USA). ^{1}H-NMR spectra were scanned on a NMR spectrophotometer (Bruker AXS Inc., Switzerland), operating at 500 MHz for ^{1}H- and 125.76 MHz for ^{13}C spectra. Chemical shifts are expressed in ppm values relative to TMS as an internal standard, using DMSO-d_6 as a solvent. Elemental analyses were done on a model 2400 CHNSO analyzer (Perkin Elmer, USA). All the values were within ± 0.4% of the theoretical values. All reagents used were of AR grade. The starting materials for sulfonamide derivatives were purchased from Sigma (USA) and were directly used for the preparation of target compounds.

Synthesis of 1,2,4-triazole-sulfonamide derivatives (1-13).

General procedure

A mixture of benzoylhydrazine (1.22 g, 0.01 mol), dimethylformamide-dimethylacetal (1.91 g, 0.01 mol)) and sulfa-drugs (0.012 mol) in dry acetonitrile (15 mL) containing acetic acid (3 mL) was refluxed for 9 h., then left to cool. The solid product formed was collected by filtration and recrystallized from dioxane to give compounds **1-13**, respectively.

4-(3-Phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (1)

Yield 66%; m.p. 191.9°C. IR (KBr, cm^{-1}): 3202, 3114 (SO_2NH_2), 3074 (CH arom.), 1615 (C=N), 1367, 1161 (SO_2). ^{1}H-NMR (DMSO-d_6): 7.4-7.9 (m, 11H, Ar-H + SO_2NH_2), 9.7 (s, 1H, CH

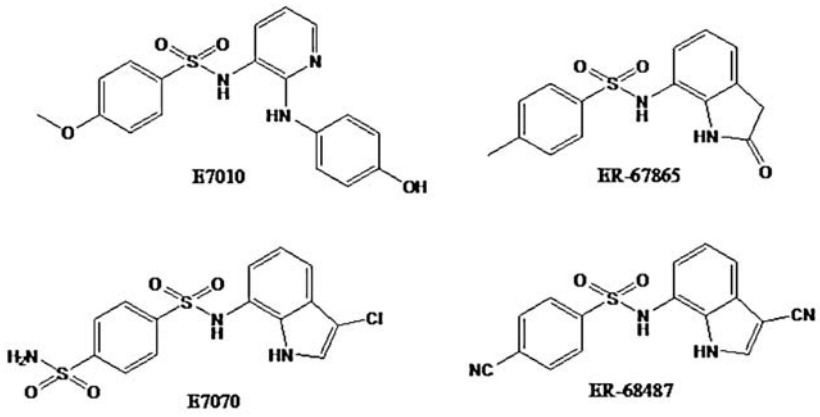


Figure 1. Drug discovery flow chart of a series of antitumor sulfonamides

triazole). ^{13}C-NMR (DMSO-d_6): 127.4 (2), 127.9 (2), 128.7 (2), 128.9 (2), 131.5 (2), 133.7 (2), 158.1, 166.3. MS m/z (%): 300 (M^+) (10.08), 74 (100). Analysis: calcd. for $C_{14}H_{12}N_4O_2S$ (300): C, 55.99; H, 4.03; N, 18.65%; found: C, 55.68; H, 4.32; N, 18.34%.

***N*-[4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) phenylsulfonyl]acetamide (2)**

Yield 81%; m.p. 233.8°C. IR (KBr, cm^{-1}): 3254 (NH), 3067 (CH arom.), 2913, 2836 (CH aliph.), 1703 (C=O), 1378, 1183 (SO_2). ^{1}H-NMR (DMSO-d_6): 2.8 (s, 3H, $COCH_3$), 7.3-8.0 (m, 9H, Ar-H), 9.3 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 19.0, 127.7 (2), 128.2 (2), 129.4 (2), 129.9, 132.9 (2), 133.1, 134.9, 137.3, 144.1, 154.2, 191.8. MS m/z (%): 342 (M^+) (8.34), 121 (100). Analysis: calcd. for $C_{16}H_{14}N_4O_3S$ (342): C, 56.13; H, 4.12; N, 16.36%; found: C, 56.41; H, 4.37; N, 16.11%.

***N*-carbamimidoyl-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (3)**

Yield 77%; m.p. 225.3°C. IR (KBr, cm^{-1}): 3343, 3222, 3132 (NH, NH_2), 3054 (CH arom.), 1373, 1152 (SO_2). ^{1}H-NMR (DMSO-d_6): 7.5 (s, 3H, NH_2 + NH imino, exchangeable with D_2O), 7.6-7.9 (m, 9H, Ar-H), 10.5 (s, 2H, SO_2NH + CH triazole), ^{13}C-NMR (DMSO-d_6):126.7 (2), 127.9 (2), 128.9 (3), 129.8 (2), 132.3 (2), 133.0, 142.8, 162.3, 166.3. MS m/z (%): 342 (M^+) (3.94), 119 (100). Analysis: calcd. for $C_{15}H_{14}N_6O_2S$ (342): C, 52.62; H, 4.12; N, 24.55%; found: C, 52.29; H, 4.46; N, 24.84%.

***N*-(3-methylisoxazol-5-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (4)**

Yield 69%; m.p. 133.8°C. IR (KBr, cm^{-1}): 3254 (NH), 3071 (CH arom.), 2943, 2854 (CH aliph.), 1602 (C=N), 1366, 1145 (SO_2). ^{1}H-NMR (DMSO-d_6): 2.3 (s, 3H, CH_3), 6.2 (s, 1H, CH isoxazole), 6.4-8.0 (m, 9H, Ar-H), 10.5 [s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 12.6, 97.5, 127.1 (2), 127.9 (2), 128.7 (2), 129.6, 129.9 (2), 132.2, 133.5, 137.0 (2), 154.4, 161.0, 170.9. MS m/z (%): 381 (M^+) (7.97), 81 (100). Analysis: calcd. for $C_{18}H_{15}N_5O_3S$ (381): C, 56.68; H, 3.96; N, 18.36%; found: C, 56.34; H, 3.59; N, 18.09%.

***N*-(3,4-dimethylisoxazol-5-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (5)**

Yield 71%; m.p. 254.2°C. IR (KBr, cm^{-1}): 3332 (NH), 3088 (CH arom.), 2932, 2822 (CH aliph.), 1589 (C=N), 1367, 1166 (SO_2). ^{1}H-NMR (DMSO-d_6): 2.1, 2.4 (2s, 6H, $2CH_3$), 6.7-8.3 (m, 9H, Ar-H), 10.0 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 6.7, 10.3, 113.0, 127.9 (2), 128.1 (2), 128.9 (2), 129.0, 129.1 (2), 132.3 (2), 132.7 (2), 153.6, 160.4, 161.5. MS m/z (%): 395 (M^+) (9.41), 174 (100). Analysis: calcd. for $C_{19}H_{17}N_5O_3S$ (395): C, 57.71; H, 4.33; N, 17.71%; found: C, 57.42; H, 4.69; N, 17.98%.

***N*-(1-phenyl-1*H*-pyrazol-5-yl)- 4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (6)**

Yield 76%; m.p. 317.6°C. IR (KBr, cm^{-1}): 3197 (NH), 3049 (CH arom.), 1584 (C=N), 1372, 1165 (SO_2). ^{1}H-NMR (DMSO-d_6): 6.8-8.7 (m, 16H, Ar-H), 9.2 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 110.3, 121.6 (2), 124.8, 125.9 (2), 126.2 (2), 127.4 (2), 128.7 (2), 128.9, 130.4 (2), 133.6, 135.5, 135.8, 142.7, 143.9 (2), 147.6, 155.8. MS m/z (%): 442 (M^+) (7.53), 92 (100). Analysis: calcd. for $C_{23}H_{18}N_6O_2S$ (442): C, 62.43; H, 4.10; N, 18.99%; found: C, 62.10; H, 4.35; N, 18.64%.

4-(3-Phenyl-4*H*-1,2,4-triazol-4-yl)-*N*-(thiazol-2-yl) benzenesulfonamide (7)

Yield 83%; m.p. 243.0°C. IR (KBr, cm^{-1}): 3231 (NH), 3100 (CH arom.), 1601 (C=N), 1365, 1149 (SO_2). ^{1}H-NMR (DMSO-d_6): 7.5-7.9 (m, 11H, Ar-H), 10.5 (s, 2H, SO_2NH + CH triazole), ^{13}C-NMR (DMSO-d_6): 122.3, 127.9 (2), 128.7 (3), 128.9 (2), 132.3 (3), 133.0 (2), 166.3 (2). MS m/z (%): 383 (M^+) (19.48), 76 (100). Analysis: calcd. for $C_{17}H_{13}N_5O_2S_2$ (383): C, 53.25; H, 3.42; N, 18.26%; found: C, 53.56; H, 3.11; N, 17.93%.

4-(3-Phenyl-4*H*-1,2,4-triazol-4-yl)-*N*-(pyridin-2-yl) benzenesulfonamide (8)

Yield 88%; m.p. 197.2°C. IR (KBr, cm^{-1}): 3363 (NH), 3082 (CH arom.), 1620 (C=N), 1393, 1159 (SO_2). ^{1}H-NMR (DMSO-d_6): 6.6-8.1 (m, 13H, Ar-H), 11.0 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 112.6, 117.5, 126.1 (2), 126.2 (3), 129.2 (3), 129.3 (2), 139.2 (2), 139.4 (2), 146.7, 152.8, 153.2. MS m/z (%): 377 (M^+) (4.32), 77 (100). Analysis: calcd. for $C_{19}H_{15}N_5O_2S$ (377): C, 60.46; H, 4.01; N, 18.56%; found: C, 60.78; H, 3.83; N, 18.19%.

4-(3-Phenyl-4*H*-1,2,4-triazol-4-yl)-*N*-(pyrimidin-2-yl) benzenesulfonamide (9)

Yield 73%; m.p. 268.8°C. IR (KBr, cm^{-1}): 3194 (NH), 3077 (CH arom.), 1598 (C=N), 1375, 1191 (SO_2). ^{1}H-NMR (DMSO-d_6): 6.0-8.4 (m, 12H, Ar-H), 11.2 (s, 2H, SO_2NH + CH triazole),^{13}C-NMR (DMSO-d_6): 112.6, 125.2 (2), 125.3 (2), 129.0 (2), 129.1 (3), 130.1 (2), 130.3 (2), 153.5, 157.7 (2),

158.7. MS m/z (%):378 (M^+) (6.74), 58 (100). Analysis: calcd. for $C_{18}H_{14}N_6O_2S$ (378): C, 57.13; H, 3.73; N, 22.21%; found: C, 57.48; H, 3.47; N, 21.88%.

***N*-(4-methylpyrimidin-2-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (10)**

Yield, 75%; m.p. 239.1°C. IR (KBr, cm^{-1}): 3280 (NH), 3039 (CH arom.), 2941, 2855 (CH aliph.), 1618 (C=N), 1359, 1164 (SO_2). ^{1}H-NMR (DMSO-d_6): 2.0 (s, 3H, CH_3), 6.6-8.3 (m, 11H, Ar-H), 11.1 (s, 2H, SO_2NH + CH triazole).^{13}C-NMR (DMSO-d_6): 23.8, 112.5, 125.5 (4), 130.5 (5), 136.7 (4), 153.4, 157.4, 158.0, 168.4. MS m/z (%): 392 (M^+) (13.62), 92 (100). Analysis: calcd. for $C_{19}H_{16}N_6O_2S$ (392): C, 58.15; H, 4.11; N, 21.42%; found: C, 57.82; H, 4.43; N, 21.11%.

***N*-(4,6-dimethylpyrimidin-2-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (11)**

Yield 78%; m.p. 248.3°C. IR (KBr, cm^{-1}): 3312 (NH), 3063 (CH arom.), 2967, 2863 (CH aliph.), 1613 (C=N), 1356, 1181 (SO_2). ^{1}H-NMR (DMSO-d_6): 2.2 (s, 6H, $2CH_3$), 6.0 (s, 1H, CH pyrimidine), 7.5-7.9 (m, 9H, Ar-H), 10.9 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 23.8 (2), 112.6, 127.1 (4), 127.9 (3), 128.9 (2), 132.3 (2), 133.0 (2), 157.2, 166.3 (3). MS m/z (%): 406 (M^+) (23.24), 79 (100). Analysis: calcd. for $C_{20}H_{18}N_6O_2S$ (406): C, 59.10; H, 4.46; N, 20.68%; found: C, 58.84; H, 4.15; N, 20.32%.

***N*-(2,6-dimethoxypyrimidin-4-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (12)**

Yield 68%; m.p. 153.6°C. IR (KBr, cm^{-1}): 3228 (NH), 3059 (CH arom.), 2933, 2862 (CH aliph.), 1622 (C=N), 1368, 1181 (SO_2), ^{1}H-NMR (DMSO-d_6): 3.80, 3.89 (2s, 6H, $2OCH_3$), 6.2 (s, 1H, CH pyrimidine), 6.4-7.5 (m, 9H, Ar-H), 7.9 (s, 2H, SO_2NH + CH triazole). ^{13}C-NMR (DMSO-d_6): 54.3, 54.8, 88.4, 113.1 (3), 122.6 (3), 124.9 (4), 129.6 (3), 154.2, 161.6, 164.3, 172.2. MS m/z (%): 438 (M^+) (19.38), 142 (100). Analysis: calcd. for $C_{20}H_{18}N_6O_4S$ (438): C, 54.79; H, 4. 14; N, 19.17%; found: C, 54.46; H, 4.43; N, 19.50%.

***N*-(5,6-dimethoxypyrimidin-4-yl)-4-(3-phenyl-4*H*-1,2,4-triazol-4-yl) benzenesulfonamide (13).**

Yield 72%; m.p. 198.1°C. IR (KBr, cm^{-1}): 3310 (NH), 3073 (CH arom.), 2974, 2832 (CH aliph.), 1611 (C=N), 1366, 1154 (SO_2). ^{1}H-NMR (DMSO-d_6): 3.6, 3.8 (2s, 6H, $2OCH_3$), 6.0-8.1 (m, 10H, Ar-H), 10.6 (s, 2H, SO_2NH + CH triazole).^{13}C-NMR (DMSO-d_6): 54.3, 60.6, 112.6 (2), 125.6 (3), 127.2 (3), 130.2 (4), 151.0 (3), 151.2, 153.9, 161.5. MS m/z (%): 438 (M^+) (2.27), 152 (100). Analysis: calcd. for $C_{20}H_{18}N_6O_4S$ (438): C, 54.79; H, 4.14; N, 19.17%; found: C, 54.40; H, 4.45; N, 19.45%.

***In vitro* anticancer evaluation**

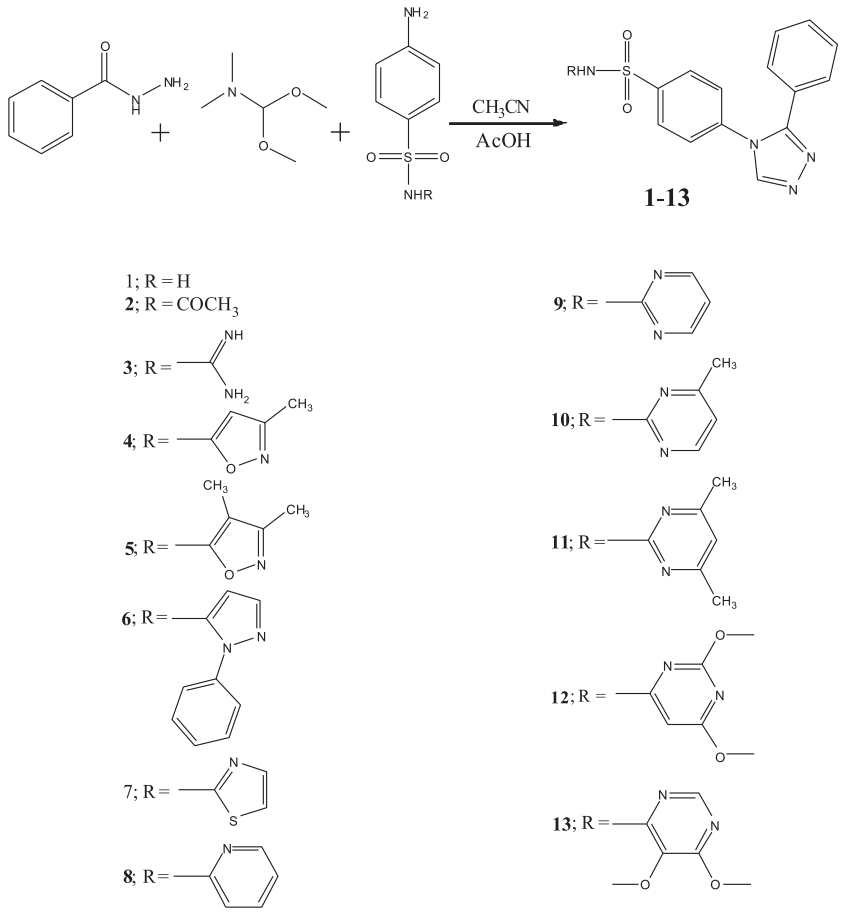
Cell culture

Human cancer cell lines HeLa (cervical), A549 (lungs) and Lovo (colorectal) were grown in DMEM

Table 1. *In vitro* anticancer screening of the synthesized compounds against four cell lines.

Compd. No.	A549 (lungs)	HeLa (cervical)	Lovo (colorectal)	MDA-231 (breast)
		IC_{50}(µg/mL)a		
1	37.01	68.96	NA	67.24
2	NA	NA	NA	NA
3	NA	NA	NA	NA
4	NA	NA	NA	NA
6	NA	NA	NA	NA
7	NA	NA	NA	NA
8	NA	NA	NA	NA
9	NA	NA	NA	NA
10	NA	NA	NA	NA
11	NA	55.71	NA	73.77
12	NA	NA	NA	NA
13	NA	NA	NA	NA
DCF	124.87	54.07	114.12	113.94

[a] All experiments were performed in triplicate, and the mean is used to calculate the IC_{50}. NA = No activity, DCF = 2',7'-dichlorofluorescein.



Scheme 1. Synthetic pathways for compounds **1**-**13**

+ GlutaMax (Invitrogen), and MDA MB321 (breast) were grown in DMEM-F12 + GlutaMax) medium (Invitrogen), supplemented with 10% heat-inactivated bovine serum (Gibco) and 1× penicillin-streptomycin (Gibco) at 37°C in a humified chamber with 5% CO_2 supply. The source of the used cancer cell lines is ATCC, Virginia, USA, and all experiments were performed in triplicate, and the mean is used to calculate the IC_{50}.

Cytotoxicity assay

The *in vitro* anticancer screening was done at Pharmacognosy Department, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia. Cells were seeded (10^5 cells/well) in 96-well flat-bottom plates (Becton-Dickinson Labware) a day before treatment and grown overnight. Compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma) and finally prepared as 1.0 mg/mL stocks, respectively, in the culture media. The final concentration of DMSO never exceeded 0.1% in the treatment doses. Four different doses of compounds (50, 25, 12.5 and 6.25 µg/mL) were further prepared by diluting the stocks in culture media, and cells were treated (in triplicate/dose). 2',7'-dichlorofluorescein (DCF) was included as standard reference drug (positive control) and untreated culture was considered as negative control. The treated cultures were further incubated for 48 h. At 48 h post-treatment, cell viability test was performed using TACS MTT Cell Proliferation and Viability Assay Kit (TACS) acc. to manufacturer's instructions. The optical density (OD) was recorded at 570 nm in a microplate reader (BioTek, ELx800) and cell survival fraction was determined. The cell survival fraction was calculated as [(A-B)/A], where A and B are the optical densities (OD) of untreated and of treated cells, respectively. The IC_{50} values of the tested compound were estimated using the best fit regression curve method in Excel.

Microscopy

A direct visual investigation was made under an inverted microscope (Optica, 40× and 100×) to

observe any morphological changes in the cells cultured with different treatment doses at 24 and 48 h.

Chemistry

The aim of this work was to design and synthesize a new series of 1,2,4-triazoles having a biologically active benzenesulfonamide moieties to evaluate their anticancer activity. There are many reported methods to synthesize substituted-1,2,4-triazole derivatives. An efficient one-pot, three components synthesis of substituted-1,2,4-triazoles has been developed by Michael (32), utilizing a wide range of substituted primary amines, acyl hydrazines, and dimethoxy-*N*,*N*-dimethylmethanamine. In this paper, the corresponding 1,2,4-triazole-sulfonamides **1**-**13** were synthesized by the Michael method: dimethoxy-*N*,*N*-dimethylmethanamine and benzoylhydrazine were reacted in acetonitrile containing acetic acid as catalyst for 9 h, and then sulfonamide derivatives were added to the mixture to obtain the corresponding 1,2,4-triazole-sulfonamides **1**-**13**, respectively (Scheme 1). The structures of the obtained products were established on the basis of microanalysis, IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data.

***In vitro* anticancer evaluation**

The synthesized compounds were evaluated for their *in vitro* anticancer activity against human lung cancer cell line (AS49-Raw), cervical (Hela) cancer cell line, colorectal cell line (Lovo) and breast cancer cell line (MDA-MB231) using 2',7'dichlorofluorescein (DCF) as reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of cancer cell lines. From the results (Table 1) it was found that 1,2,4-triazole **1** having free sulfonamide SO_2NH_2 with unsubstituted phenyl ring exhibited higher activity against lung and breast cancer cell lines with IC_{50} values (37.01, 67.24 µg/mL) than the positive control DCF with IC_{50} values (124.87 and 113.94 µg/mL). Also, it was found that compound **1** exhibited a remarkable activity against Hela cell line with IC_{50} value (68.96 µg/mL). In addition, compound **1** revealed no activity against Lovo (colorectal) cell line. On the other hand, 1,2,4-triazole **11** containing sulfonamide with dimethylpyrimidine showed higher activity with IC_{50} value (73.77 µg/mL) against breast cancer cell line compared with DCF with IC_{50} value (113.94 µg/mL). Compound **11** with IC_{50} value (55.71 µg/mL) is nearly as active as DCF with IC_{50} value (54.07 µg/mL) as positive control against HeLa cell line.

CONCLUSION

In this work, a novel 1,2,4-triazoles-sulfonamides hybrids were synthesized and their *in vitro* anticancer activity was evaluated on four human tumor cancer cell lines. Among the tested compounds, two candidates **1** and **11** were the most potent in this study, which showed higher activity than the reference drug 2',7'-dichlorofluorescein (DCF). The active compounds could be considered as useful templates for further development to obtain more potent anticancer agent(s).

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SYNTHESIS AND ANTIMICROBIAL ACTIVITY OF 5-SUBSTITUED 4-THIAZOLIDINONES WITH SULFANILAMIDE PHARMACOPHORE

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Abstract: After incorporation pharmacophores of allylamine and sulfanilamide into 4-thiazolidinone's ring – no antimicrobial activity was determined. This outcome stimulated synthesis of new group - 5-substituted 4-thiazolidinones. In the literature it is noted that the fragment of aldehyde in 5 position of 4-thiazolidinone's ring should give or increase biological activity. So, it was decided to incorporate fragment of aldehyde into 4-thiazolidinone's ring together with sulfanilamide pharmacophore, investigate antimicrobial activity and compare it with initial compounds – sulfanilamides. It was established that new compounds suppressed growth of *S. aureus*, *E. coli*, *B. subtilis*, *P. mirabilis*, *C. albicans*. Sulfanilamide, sulfapyridine and/or 2-chlorobenzaldehyde were incorporated into the structure of the most active compounds. It was concluded that synthesis of 4-thiazolidinones substituted by aldehyde in 5 position and sulfanilamide in 2 position are not potential antimicrobial agents.

Keywords: antimicrobial activity, 4-thiazolidinones, sulfanilamide.

Currently, more than 200 antimicrobial agents are used in clinical practice for the treatment of infectious diseases. However, infectious diseases are still one of the most common causes of death (1, 2). Due to growing resistance of microorganisms, it is necessary to continue the search for new active substances, because the infections caused by resistant bacteria in the future may become the cause of epidemic which would be dangerous for many people (3, 4).

The analysis of the structure of compounds synthesized previously showed that 4-thiazolidinone derivatives with sulfonamide pharmacophore are more active against bacteria than sulfanilamides themselves, also they are characterized by antifungal activity (5, 6). Recently, the various synthetic reactions using sulfonamides fragments are described with expectation of products with antifungal activity and decreased toxicity (7-9).

Previous synthesis of similar compounds (incorporation pharmacophores of allylamine and sulfanilamide into 4-thiazolidinone's ring) without antimicrobial activity, induced synthesis of new group - 5-substituted 4-thiazolidinones (10). In 5-position of 4-thiazolidones the methylene group is enough active, so most new compounds are synthesized by modification of this position. (11, 12). Knoevenagel condensation of the C-5 active methylene of 4-thiazolidones with oxo compounds under basic catalysis yielding of 5-arylidene derivatives constitutes an efficient way to new biologically active substances. 5-Arylidene-4-thiazolidones display a wide spectrum of pharmacological properties. (12-15). Also, the aldehyde fragment into 5 position of 4-thiazolidinone's ring should give or increase biological activity. (15, 16).

EXPERIMENTAL

Materials and Metods

New compounds were synthesized at Department of Pharmaceutical Chemistry in Lithuanian University of Health Sciences. All reactions were monitored by TLC (Merck Kieselgel 60

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F_{254}). Melting points were determined with Kofler's melting point apparatus and are uncorrected. Elemental analyses were performed by analyzator Gerhardt Vapodest 20 (nitrogen) and by Schoniger's method (sulfur). Infrared (IR) spectra were recorded on spectrometer Spectrum 100 FT-IR ("Perkin-Elmer"). The NMR spectra were taken on a Varian Unity Inova spectrometer (300 MHz for ^{1}H). Purity was checked at Department of Analytical and Toxicological Chemistry in Lithuanian University of Health Sciences by HPLC method (chromatograph Waters 2695 with photodiode detector Waters 996 PDA, analytes were separated using C18 Hypersil „Thermo Scientific" analytical column (5 μm, 250 × 4.6 mm) with precolumn).

CHEMISTRY

Synthesis of 5-substituted-2-methylmercaptothiazolidin-4-ones (intermediates)

2-Methylrhodanine (0.04 mol) dissolved in glacial acetic acid (30 mL) and the appropriate aldehyde (0.05 mol) was added (Fig. 1). Ammonium acetate (0.005 mol) was used as catalyst. The reaction mixture was stirred for 5-20 min at 60°C. Then, the solid separated was filtered,

Table 1. Characterization data of new compounds.

Compound no.	Molecular formula and molecular weight	Yield (%)	Melting point, °C (solvent)	Calculated, % **found, %**	
				N	**S**
1	$C_{21}H_{17}FN_4O_4S_2$ 472.52	80	318–320 (CH_3COOH)	11.86 **11.6**	13.57 **13.3**
2	$C_{21}H_{15}FN_4O_3S_2$ 454.51	88	326–328 (CH_3COOH)	12.33 **12.6**	14.11 **14.5**
3	$C_{21}H_{16}FN_5O_4S_2$ 485.52	91	313–314 (CH_3COOH)	14.42 **14.8**	13.21 **13.5**
4	$C_{22}H_{18}FN_5O_3S_2$ 483.55	89.6	300–302 (CH_3COOH)	14.48 **14.9**	13.26 **13.6**
5	$C_{21}H_{14}Cl_2N_4O_3S_2$ 505.40	90.1	218–220 (CH_3COOH)	11.09 **10.9**	12.69 **12.5**
6	$C_{21}H_{18}N_4O_5S_2$ 470.53	77	348–350 (CH_3COOH)	11.91 **11.6**	13.63 **13.3**
7	$C_{12}H_{17}ClN_4O_4S_2$ 488.98	82	319–320 (CH_3COOH)	11.46 **11.3**	13.11 **13.0**
8	$C_{21}H_{16}ClN_5O_4S_2$ 501.97	98	310–312 (CH_3COOH)	13.95 **14.1**	12.78 **12.9**
9	$C_{22}H_{20}N_6O_6S_2$ 528.56	73.2	322–324 (dioxane)	15.09 **15.8**	12.13 **12.6**
10	$C_{21}H_{17}BrN_4O_4S_2$ 533.43	93.8	325–327 (CH_3COOH)	10.50 **10.9**	12.02 **12.1**
11	$C_{22}H_{18}BrN_5O_3S_2$ 544.45	55.2	318–320 (CH_3COOH)	12.86 **13.1**	11.78 **12.0**
12	$C_{21}H_{15}BrN_4O_3S_2$ 515.41	90.3	345–348 (CH_3COOH)	10.87 **10.7**	12.44 **12.3**
13	$C_{21}H_{17}FN_4O_4S_2$ 472.51	84.5	324-326 (CH_3COOH)	11.86 **11.52**	13.57 **13.01**
14	$C_{22}H_{18}FN_5O_3S_2$ 483.54	89.6	306-308 (CH_3COOH)	14.48 **15.02**	13.26 **13.40**
15	$C_{21}H_{16}FN_5O_4S_2$ 485.52	95.9	317–319 (CH_3COOH)	14.42 **14.2**	13.21 **13.0**
16	$C_{21}H_{15}FN_4O_3S_2$ 454.51	88.2	323–325 (CH_3COOH)	12.33 **12.21**	14.11 **14.0**
17	$C_{19}H_15N_5O_7S_2$ 489.49	81.6	348–350 (CH_3COOH)	14.31 **14.5**	13.10 **13.2**

washed with water, ethanol, ether and recrystallized from acetone.

Synthesis of 5-substituted-2-sulfanilamide-thiazolidin-4-ones

Intermediate (5-substituted-2-methylmercaptothiazolidin-4-one) (0.003 mol) dissolved in glacial acetic acid (10-40 mL) or dioxane (**9**, 30 mL) and the appropriate sulfanilamide (0.004-0.005 mol) was added. The reaction mixture was heated at 90°C for 3 h (**4**, **6**, **7**, **8**, **9**) or 4 h (**5**, **10**, **11**, **12**, **13**, **14**, **15**, **16**, **17**) or 6 h (**1**, **2**) or 24 h (**3**). Then, the solid separated was filtered, washed with water, ethanol, ether and recrystallized from glacial acetic acid or dioxane (**9**).

Determination of antimicrobial activity

Antimicrobial activity was tested at Department of Microbiology in Lithuanian University of Health Sciences.

Antimicrobial susceptibility tests

Antimicrobial activity was tested *in vitro* in Mueller-Hinton agar (Mueller-Hinton II Agar, BBL, Cockeysville, USA). Antimicrobial activity of new compounds was tested in standard bacteria cultures: *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 33499, *Proteus mirabilis* ATCC 12459, *Bacillus subtilis* ATCC 6633 and standard fungal culture: *Candida albicans* ATCC 60193. These bacterial and fungal strains were selected for research because of different structure and functions. Also they are used as standard microorganisms for determination of antimicrobial activity.

Preparation of standard microorganism cultures

Standard bacteria and fungal cultures were cultivated 20–24 h on Mueller-Hinton agar at 35–37°C temperature. Bacterial and fungal suspensions were prepared from cultivated cultures in physiological solution according to turbidity standard 0.5 McFarland.

Preparation of test compounds solutions

Test compounds were dissolved in dimethyl sulfoxide (20 mg/mL) and then diluted to obtain

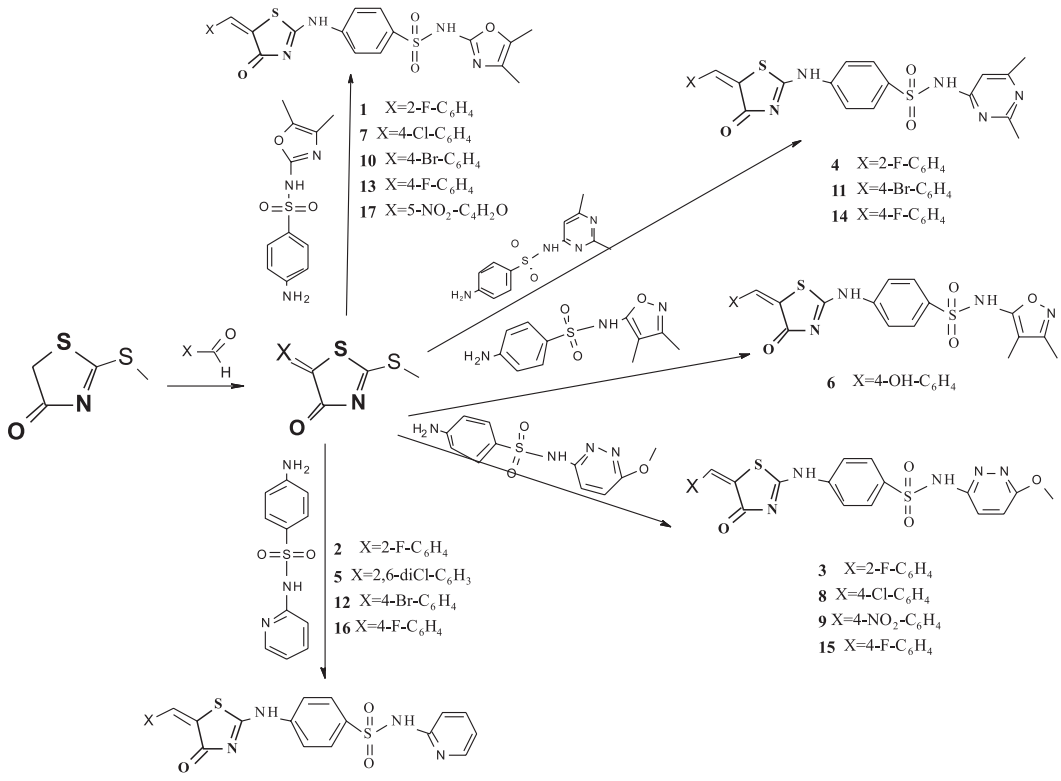

Figure 1. Synthesis of final compounds

final concentration ranging from 1 to 1000 μg/mL. Diluted solutions were mixed with 10 mL of Mueller-Hinton agar. Petri plates were incubated for 20–24 h at 35–37°C. The minimal concentration of antimicrobial (antifungal) compound that prevents any growth of tested bacteria (fungi) was indicated

Table 2. Spectral data of new compounds.

Compd. no.	IR (cm^{-1})	^{1}H NMR (300 MHz, DMSO-d_6, δ, ppm)
1	1725 (C=O). 710 (-CH=)	2.23 (3H, s, CH_3-), 2.29 (3H, s, CH_3-), 7.17 (1H, m, ArH), 7.41 (1H, m, ArH), 7.61 (1H, s, -CH=), 7.63 (1H, m, ArH), 7.79 (1H, m, ArH), 8.23 – 7.97 (4H, m, ArH)
2	1710 (C=O). 711 (-CH=)	7.16 (1H, m, ArH), 7.22 (1H, m, ArH), 7.31 (1H, m, ArH), 7.61 (1H, s, -CH=), 7.69 (1H, m, ArH), 7.74 (1H, m, ArH), 7.79 (1H, m, ArH), 7.97 (1H, m, ArH), 7.97 (1H, m, ArH), 8.05 (1H, m, ArH), 8.23 (1H, m, ArH), 8.23 (1H, m, ArH), 8.48 (1H, m, ArH)
3	1732 (C=O). 715 (-CH=)	3.83 (3H, s CH_3-), 7.17 (1H, m, ArH), 7.24 (1H, d, ArH), 7.30 (1H, m, ArH), 7.35 (1H, d, ArH), 7.61 (1H, s, -CH=), 7.63 – 7.79 (2H, m, ArH), 8.23 – 7.97 (4H, m, ArH)
4	1710 (C=O). 689 (-CH=)	2.58 (3H, s, CH_3-), 2.59 (3H, s, CH_3-), 6.576 (1H, s, -CH=), 7.61 (1H, s, -CH=), 7.79 – 7.32 (4H, m, ArH), 8.23 – 7.97 (4H, m, ArH)
5	1720 (C=O). 742 (-CH=). 658 (C-Cl)	7.35 (1H, dd, ArH), 7.38 (1H, dd, ArH), 7.45 (1H, m, ArH), 7.57 (1H, dd, ArH), 7.74 (1H, m, ArH), 7.79 (1H, s, -CH=), 7.96 (1H, m, ArH), 7.97 (2H, m, ArH), 8.23 (2H, m, ArH), 8.61 (1H, m, ArH)
6	3256 (OH) 1734 (C=O). 730 (-CH=).	2.23 (3H, s, CH_3-), 2.29 (3H, s, CH_3-), 7.49 – 6.95 (4H, m, ArH), 7.66 (1H, s, -CH=), 8.24 – 7.97 (4H, m, ArH)
7	1740 (C=O). 710 (-CH=) 658 (C-Cl)	2.23 (3H, s, CH_3-), 2.29 (3H, s, CH_3-), 7.57 – 7.50 (4H, m, ArH), 7.71 (1H, s, -CH=), 8.24 – 7.97 (4H, m, ArH)
8	1742 (C=O) 705 (-CH=) 662 (C-Cl)	3.84 (3H, s, CH_3-), 7.10 (1H, d, ArH), 7.51 (1H, m, ArH), 7.56 (2H, m, ArH), 7.60 (1H, d, ArH), 7.71 (1H, s, -CH=), 7.74 (1H, m, ArH), 8.26 – 7.99 (4H, m, ArH)
9	1715 (C=O) 709 (-CH=) 1562 (NO_2)	3.83 (3H, s, CH_3-), 7.35 – 7.24 (2H, d, -CH=), 7.80 (1H, s, -CH=), 7.86 – 7.85 (2H, m, ArH), 7.95 (2H, m, ArH), 8.14 – 8.12 (2H, m, ArH), 8.30 (2H, m, ArH)
10	1714(C=O) 713 (-CH=) 536 (C-Br)	2.23 (3H, s, CH_3-), 2.29 (3H, s, CH_3-), 7.68 (1H, s, -CH=), 7.73 – 7.30 (4H, m, ArH), 8.24 – 7.97 (4H, m, ArH)
11	1723 (C=O) 721 (-CH=) 545 (C-Br)	2.58 (3H, s, CH_3-), 2.59 (3H, s, CH_3-), 6.58 (1H, s, ArH), 7.68 (1H, s, -CH=), 7.73 – 7.30 (4H, m, ArH), 8.25 – 7.97 (4H, m, ArH)
12	1734 (C=O) 695 (-CH=) 539 (C-Br)	7.46 (2H, m, ArH), 7.47 (1H, m, ArH), 7.49 (1H, m, ArH), 7.68 (1H, s, -CH=), 7.73 (1H, m, ArH), 7.91 (1H, m, ArH), 7.96 (1H, m, ArH), 7.98 (2H, m, ArH), 8.23 (2H, m, ArH), 8.62 (1H, m, ArH)
13	1712 (C=O) 700 (-CH=)	2.23 (3H, s, CH_3-), 2.27 (3H, s, CH_3-), 7.23 (2H, m, ArH), 7.70 (1H, s, -CH=), 7.82 (2H, m, ArH), 8.24–7.97 (4H, m, ArH)
14	1741 (C=O) 699 (-CH=)	2.58 (s, 3H, CH_3-), 2.59 (s, 3H, CH_3-), 6.58 (s, 1H, ArH), 7.22 (2H, m, ArH), 50 (s, 1H, =CH-), 7.7-7.8 (2H, m, ArH), 7.96 (2H, m, ArH), 8.16-8.19 (2H, m, ArH)
15	1738 (C=O) 724 (-CH=)	3.83 (3H, s, CH_3-), 7.24 (1H, d, ArH), 7.33 (2H, m, ArH), 7.35 (1H, d, ArH), 7.70 (1H, s, -CH=), 7.82 (2H, m, ArH), 8.24 – 7.97 (4H, m, ArH)
16	1720(C=O) 715 (-CH=)	7.04 (1H, m, ArH), 7.26 (2H, m, ArH), 7.29 (1H, m, ArH), 7.70 (1H, s, -CH=), 7.81 (1H, m, ArH), 7.87 (1H, m, ArH), 7.97 (2H, m, ArH), 7.98 (1H, m, ArH), 8.23 (2H, m, ArH), 8.60 (1H, m, ArH)
17	1718 (C=O) 716 (-CH=) 1550 (NO_2)	2.23 (3H, s, CH_3-), 2.29 (3H, s, CH_3-), 7.14 (1H, d, -CH=), 7.51 (1H, d, -CH=), 7.71 (1H, s, -CH=), 7.95 – 8.16 (4H, m, ArH)

Table 3. Antimicrobial data of new compounds.

Microorganism / Compound	Minimal inhibitory concentration, μg/mL					
	S. aureus	*E. faecalis*	*E. coli*	*B. subtilis*	*P. mirabilis*	*C. albicans*
1	250 ± 25	-	150 ± 25	250 ± 25	250 ± 25	300 ± 50
2	-	300 ± 50	150 ± 25	250 ± 25	250 ± 25	-
3	250 ± 25	-	150 ± 25	500 ± 50	250 ± 25	300 ± 50
4	250 ± 25	150 ± 25	150 ± 25	250 ± 25	250 ± 25	500 ± 50
5	25 ± 5	25 ± 5	25 ± 5	20 ±5	250 ± 25	25 ± 5
6	100 ± 10	-	1 ± 0.1	300 ± 50	250 ± 25	300 ± 50
7	250 ± 25	-	150 ± 25	500 ± 50	250 ± 25	300 ± 50
8	250 ± 25	-	150 ± 25	-	250 ± 25	300 ± 50
16	250 ± 25	-	50 ± 10	100 ± 10	-	50 ± 10
17	400 ± 50	-	400 ± 50	500 ± 50	-	-
Sulfamethoxy-pyridazine	300 ± 50	100 ± 10	100 ± 10	50 ± 10	100 ± 10	500 ± 50
Sulfamoxole	-	-	500 ± 50	100 ± 10	300 ± 50	500 ± 50
Sulfapyridine	-	-	500 ± 50	100 ± 10	-	-
Sulfisomidine	-	-	300 ± 50	100 ± 10	500 ± 50	-
Sulfafurazole	-	-	300 ± 50	100 ± 10	100 ± 10	-

as minimal inhibitory concentration (MIC). Sulfanylamides (sulfamethoxypyridazine, sulfamoxole, sulfapyridine, sulfisomidine, sulfafurazole) were used as standard drugs.

RESULTS

All new compounds were synthesized successfully. Obtained compounds are yellow, orange or brown crystals, insoluble in water, slightly soluble in alcohol, glacial acetic acid, soluble in DMSO and DMF. It was determined that purity of new compounds is 98-99%. The structures of new compounds were confirmed by elemental analysis (we have determined quantity of nitrogen (N) and sulfur (S) in each compound) and spectral analysis (IR and ^{1}H NMR). All characterization data (molecular formula and molecular weight, yield, melting point, quantity of nitrogen (N) and sulfur (S)) are shown in Table 1, spectral data are shown in Table 2.

The results of antimicrobial activity showed that new compounds can be characterized as antimicrobial and antifungal agents. Antimicrobial activity of new compounds was compared with initial compounds – sulfanilamides, which antimicrobial activity was tested at the same conditions as new compounds. It was concluded that 7 new compounds (**9**, **10**, **11**, **12**, **13**, **14**, **15**) were inactive at all. No one of the tested compounds showed activity against *Pseudomonas aeruginosa* (MIC > 1 mg/mL) and *Proteus mirabilis* (MIC > 1 mg/mL). The inhibitory concentrations for *S. aureus, E. coli, B. subtilis, P. mirabilis, C. albicans* were the lowest. Data of antibacterial and antifungal activity are shown in Table 3.

DISCUSSION

Derivatives of 2-thioxo-4-thiazolidinone exist in tautomeric forms: 2-thioxo-4-thiazolidinone, 4-hydroxy-2(5H)-thiazolethione, 2-mercapto-4(5H)-thiazolone, 4-hydroxy-2(3H)-thiazolethione and 2-mercapto-4-thiazolol (17). 2-Alkylthio-4-thiazolidinone can be obtained by alkylation from 2-mercapto-4(5H)-thiazolone. Amination of 2-alkylthio-4-thiazolidinone goes faster than with rhodanine. Also, higher yield and purer compounds are obtained. 2-Alkylrhodanine can be obtained by reaction of rhodanine and halogen alkane in the presence of base (NaOH, KOH, NaH, EtN_3) (12). In our experiments, 2-methylrhodanine was synthesized according to the methodology suggested by Tarasevicius. This method allows to obtain a higher yield of 2-methylrhodanine (82–91%) (15). During the condensation of 2-methylrhodanine with various aldehydes, the reaction proceeded by heating initial substances in glacial acetic acid at 60^{o}C. It was

found that increasing the temperature makes the reaction time shorter, but the yield is lower (15). Ammonium acetate or sodium acetate was used as catalyst. Catalyst for the reaction was chosen considering the results, obtained from the results described in previous works (10, 15), and our experiments.

2-Alkylthio-4-thiazolidinone can react with aromatic amines to form derivatives of 2-imino-4-thiazolidinone (15, 18). 5-Substituted 4-thiazolidinone derivatives, having sulfanilamide pharmacophore were synthesized according to Knoevenagel's condensation, which is based on addition of an active hydrogen compound to carbonyl group followed by dehydration reaction (12, 19). The process of reaction between 5-substituted rhodanine and sulfanilamide was observed using the lead acetate indicator. It was determined that the time of reaction depends on reagents proportion and structure of sulfanilamide. Also it was found that the excess of one of the initial reaction substrates, increases the speed of reaction.

Antimicrobial activity of compounds was compared with initial compounds – sulfanilamides. Five different sulfanilamides: sulfamethoxypyridazine, sulfamoxole, sulfafurazole, sulfisomidine and sulfapyridine were used in synthesis. Sulfamethoxypyridazine showed the highest antibacterial activity (MIC = 50–300 μg/mL), but all sulfanilamides had not shown antifungal activity (MIC = 500 μg/mL). Sulfapyridine itself was active only against *B. subtilis* (MIC = 100 μg/mL) and showed insignificant activity against *E. coli* (MIC = 500 μg/mL). However, after incorporation into 4-thiazolidone ring, its antimicrobial activity was increased. The most active compounds (**2**, **5**, **16**) had fragment of sulfapyridine (MIC = 25–300 μg/mL).

Influence of an aldehydes was studied from a viewpoint of a functional group, situated in benzaldehyde ring. The compounds having halogen formed the largest group, their MIC = 25–500 μg/mL. Because various halogens have been introduced into the structure of compounds, their various quantity and position in the molecule and the influence of this differences on activity have been compared too. It has been established that compounds activity depends on halogen nature (**7** – chlorine, **10** – bromine, **13** – fluorine). The best activity showed compounds with chlorine (**7**), as compounds having bromine and fluorine (**10**, **13**) were inactive. Also, the compound having two chlorine atomes in its structure (**5**), was more active (MIC = 25–250 μg/mL) than compounds, in which structure only one chlorine atom was present (**7**, **8**). However, in this case it is not possible to suggest that incorporation of the second chlorine atom increases antibacterial activity, because there are fragments of different sulfanilamides in these structures. Because we had compounds with 4 different sulfanilamides and 2- or 4-fluorobenzaldehyde substitutents, we could compare the influence of halogen position in benzaldehyde ring. It was found that change of halogens position (from 2 for 4) had influence on antibacterial activity. This activity was decreased (**2**, **16**) or disappeared (**1**, **3, 4, 13, 14, 15**). Sulfapyridine derivatives (after change of halogen position from 2 for 4) assumed an activity against *S. aureus* or *C. albicans*. The influence on antifungal activity was similar.

Comparing antimicrobial activity of 3 compounds (**3**, **8**, **9**), it was determined that nitro group 2–3 times decreased the antibacterial activity, either 1.5 time antifungal activity. Compound having nitrofurane substitutent (**17**) suppressed the growing of investigated bacteria only in high concentrations (*S. aureus* and *E. coli* – MIC = 400 μg/mL, *B. subtilis* – MIC = 500 μg/mL) or absolutely did not affect them (MIC > 500 μg/mL). Either this compound hadn't antifungal activity.

In general, the incorporation of the aldehyde into the structure of new compounds increased the antimicrobial activity ($p < 0.05$). This confirmed the literature data of previous studies (15, 16, 20), but in most cases activity was not greater than 100 μg/mL (with the exception of compound **5**, which MIC varies within 25-250 μg/mL). After evaluation of impact of tested aldehydes, it can be concluded that the best way is to introduce 2-chloroaldehydes, if antimicrobial activity is expected.

CONCLUSION

We concluded that synthesis of 4-thiazolidinones substituted by aldehyde in 5 position and sulfanilamide in 2 position are not potential antimicrobial agents.

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SYNTHESIS, BIOLOGICAL EVALUATION AND MOLECULAR DOCKING STUDIES OF AROMATIC SULFONAMIDE DERIVATIVES AS ANTI-INFLAMMATORY AND ANALGESIC AGENTS

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Abstract: In this study, [4-(*N*-substituted sulfamoyl)phenyl]carbonohydrazonoyl dicyanides **3a-c** were synthesized and condensed with various hydrazine hydrate derivatives to produce the corresponding 3,5-diaminopyrazole derivatives **4-9**, respectively. Furthermore, condensation of **3b** with α-naphthol, urea and thiourea yielded the pyrimidine derivatives **10** and **11a,b**, respectively. Also, condensation of **3b** with hydroxylamine hydrochloride produced the isoxazole derivative **12**. Treatment of **3b** with different secondary amines afforded the piperidine and piperazine derivatives **13a-c**, respectively, while its condensation with diamines yielded the corresponding diazepine, benzodiazepine and benzooxazepine derivatives **14-16**. Reaction of **3b** with malononitrile or diazonium salt **2b** with MND followed by treatment with malononitrile afforded the pyrido-pyridazine derivative **18**. Anti-inflammatory and analgesic evaluation of some of the synthesized compounds as representative examples exhibited equipotent activity to that of the reference drug celecoxib. The ulcerogenic potential of the tested derivatives showed a complete safety profile on G.I.T. system. Molecular docking studies showed that the tested compounds induced good fitting and forming different hydrogen bonds with the amino acid residues at the active sites of COX-2 enzyme.

Keywords: sulfonamides, pyrazole, anti-inflammatory, analgesic, ulcerogenic activity, molecular docking

The sulfonamide group is considered as a pharmacophore which plays a very important role as a key constituent of a number of drugs in clinical use such as carbutamide, darunavir and sulfamethoxazole (1-3). In addition, many sulfonamide derivatives have been reported to produce antimicrobial (4-8), antiviral (9) and herbicidal activities (10). Other sulfonamide derivatives are considered as anti-inflammatory (11-13), diuretic (14), antiglaucoma (15) and anticonvulsant agents (16). Also, sulfonamides have extensive applications in cancer chemotherapy (17, 18) and some other applications produce carbonic anhydrase and protease inhibiting effect (19, 20). Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly prescribed medications for the treatment of pain, fever, and inflammation, however, their clinical usefulness is still restricted due to their gastrointestinal side effects as gastric irritation, ulceration, bleeding and in some cases may lead to life-threatening conditions (21). Based on these side effects, it has been suggested that selective COX-2 inhibitors (coxibs) may act as safer NSAIDs devoid of ulcerogenic side effects.

Therefore, the development of new anti-inflammatory and analgesic active drugs with less ulcerogenic side effects is still a challenging target for the pharmaceutical industry (21, 22). Based on the previous findings, this study is aiming to synthesize safer and effective anti-inflammatory drug candidates. Our strategy is directed towards design, synthesis and anti-inflammatory and analgesic evaluation of new structure leads comprising mainly the substituted phenylsulfamyl counterpart conjugated

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with various heterocyclic functionalities of documented anti-inflammatory and analgesic activity such as pyrazole, pyrimidine, isoxazole, diazepine, benzodiazepine, benzoxazepine and pyrido-pyridazine (23-28) ring systems. In addition, a molecular docking study was performed in an attempt to understand the binding mode of the new derivatives to the binding site of COX-2.

EXPERIMENTAL

Chemistry

All melting points are uncorrected and were taken in open capillary tubes using electrothermal apparatus 9100. Elemental microanalyses were carried out at Microanalytical Unit, Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt, using Vario Elementar apparatus and were found within ±0.4% of the theoretical values. Infrared spectra were recorded on a Jasco FT/IR-6100, Fourier transform, infrared spectrometer at cm^{-1} scale using KBr disc technique at Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt. ^1H NMR spectra were determined by using a JEOL AS-500 NMR spectrometer at Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt, chemical shifts are expressed in δ (ppm) downfield from TMS as an internal standard. The mass spectra were measured with a GC MS-Qp1000EX Shimadzu apparatus, Cairo University, Cairo, Egypt. Follow up of the reactions and checking the purity of the compounds were made by TLC on silica gel-precoated aluminium sheets (Type 60, F254, Merck, Darmstadt, Germany) using chloroform/methanol (10 : 1, v/v) mobile phase and the spots were detected by exposure to UV lamp at λ_{254} nanometer for few seconds and by iodine vapor.

The chemical names given for the prepared compounds are according to the IUPAC system. *N*-methylsulfanilamide, *N*-butylsulfanilamide and *N*-cyclohexylsulfanilamide were obtained from commercial sources and were used without further purification. *p*-Chlorobenzoyl hydrazine was prepared according to the reported method (29).

General procedure for the synthesis of [4-(*N*-substituted sulfamoyl)phenyl]carbon-hydrazonoyl dicyanides 3a-c

A solution of sodium nitrite (0.9 g, 0.013 mol) in distilled water (5 mL) was added portionwise to an ice cold solution of sulfanilamide derivatives namely: *N*-methylsulfanilamide, *N*-butylsulfanilamide and/or *N*-cyclohexylsulfanilamide **1a-c** (0.01 mol) in concentrated hydrochloric acid (2.5 mL) and distilled water (5 mL). This solution was added portionwise to a well-stirred cold solution of malononitrile (0.66 g, 0.01 mol) in ethanol (30 mL) containing sodium acetate (0.9 g, 0.011 mol). The reaction mixture was kept in an ice bath for 2 h and the formed precipitate was filtered, washed several times with water, dried and recrystallized from ethanol to give title compounds **3a-c**, respectively.

[4-(*N*-Methylsulfamoyl)phenyl]carbonohydrazonoyl dicyanide (3a)

Yield 63%; m.p. 171-172°C; IR (KBr, cm^{-1}): 3245, 3195 (2NH), 2950, 2857 (CH-aliph.), 2216 (CN), 1329, 1160 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 2.34 (s, 3H, CH_3), 6.59 (d, 2H, Ar-H), 7.42 (d, 2H, Ar-H), 8.54, 12,95 (2br s, 2H, 2NH, D_2O exchangeable); MS, m/z (%): 263 [M^+] (39), 233 [M^+ - CH_3NH] (100); Analysis: calcd. for $C_{10}H_9N_5O_2S$ (263.28): C, 45.62; H, 3.45; N, 26.60; S, 12.18%; found: C, 45.49; H, 3.61; N, 26.78; S, 11.99%.

[4-(*N*-Butylsulfamoyl)phenyl]carbonohydrazonoyl dicyanide (3b)

Yield 91%; m.p. 184-186°C; IR (KBr, cm^{-1}): 3289, 3249 (2NH), 2931, 2868 (CH-aliph.), 2227 (CN), 1330, 1158 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.75 (t, 3H, CH_3), 1.17 (m, 2H, CH_2), 1.29 (m, 2H, CH_2), 2.68 (t, 2H, CH_2), 7.55 (d, 2H, Ar-H), 7.76 (d, 2H, Ar-H), 7.53, 13.22 (2br s, 2H, 2NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.40 (CH_3), 19.16, 31.01 ($2CH_2$), 42.15 (N-CH_2), 109.70, 114.01 (2CN), 86.72, 116.66-144.50 (7 Ar-C); MS, m/z (%): 305 [M^+] (46), 233 [M^+ - $C_4H_{10}N$] (100); Analysis: calcd. for $C_{13}H_{15}N_5O_2S$ (305.36): C, 51.13; H, 4.94; N, 22.94; S, 10.50%; found: C, 50.99; H, 4.82; N, 23.08; S, 10.41%.

[4-(*N*-Cyclohexylsulfamoyl)phenyl]carbonohydrazonoyl dicyanide (3c)

Yield 53%; m.p. 207-209°C; IR (KBr, cm^{-1}): 3286, 3256 (2NH), 2931, 2861 (CH-aliph.), 2226 (CN), 1337, 1168 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 1.27-1.76 (m, 10H, 5(CH_2) cyclohexyl protons), 2.57-2.67 (1H, m, -NC*H* cyclohexyl proton), 7.25 (d, 2H, Ar-H), 7.76 (d, 2H, Ar-H), 7.91, 13.14 (2br s, 2H, 2NH, D_2O); MS, m/z (%): 331 [M^+] (26), 249 [M^+ - C_6H_{10}] (18), 233 [M^+ - $C_6H_{12}N$] (100); Analysis: calcd. for $C_{15}H_{17}N_5O_2S$ (331.39): C, 54.36; H, 5.17; N, 21.13; S, 9.68%; found: C, 54.12; H, 5.07; N, 20.99; S, 9.79%.

General procedure for the synthesis of *N*-substituted-4-[(3,5-diamino-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamides 4a,b

A mixture of the hydrazone derivatives **3b**,**c** (0.01 mol) and hydrazine hydrate (98%, 2.0 mL, 0.02 mol) in absolute ethanol (20 mL) was refluxed for 2 h. On cooling, the formed precipitate was filtered, dried and recrystallized from ethanol to give title compounds **4a**,**b**, respectively.

***N*-Butyl-4-[(3,5-diamino-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (4a)**

Yield 77%; m.p. 168-169°C; IR (KBr, cm^{-1}): 3410, 3294, 3178 (2NH, $2NH_2$), 2929, 2869 (CH-aliph.), 1323, 1156 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.75 (t, 3H, CH_3), 1.19 (m, 2H, CH_2), 1.29 (m, 2H, CH_2), 2.68 (t, 2H, CH_2), 5.98, 6.55 (2br s, 4H, $2NH_2$, D_2O exchangeable), 7.44, 10.84 (2br s, 2H, NH, D_2O exchangeable), 7.70 (d, 2H, Ar-H), 7.77 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.42 (CH_3), 19.17, 31.0 ($2CH_2$), 42.18 (N-CH_2), 115.80-137.08 (8 Ar-C), 156.15 (C=N); MS, m/z (%): 337 [M^+] (85), 249 [M^+ - $C_4H_{10}NO$] (18), 233 [M^+ - $C_4H_{10}NO_2$] (100); Analysis: calcd. for $C_{13}H_{19}N_7O_2S$ (337.40): C, 46.28; H, 5.68; N, 29.06; S, 9.50%; found: C, 46.12; H, 5.57; N, 28.91; S, 9.71%.

***N*-Cyclohexyl-4-[(3,5-diamino-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (4b)**

Yield 46%; m.p. 98-100°C; IR (KBr, cm^{-1}): 3405, 3259, 3125 (2NH, $2NH_2$), 2918, 2852 (CH-aliph.), 1331, 1151 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 1.25-1.74 (m, 10H, $5CH_2$-cyclohexyl protons), 2.58-2.69 (1H, m, -NCH-cyclohexyl proton), 5.46, 6.75 (2br s, 4H. $2NH_2$, D_2O exchangeable), 7.23, 12.33 (2br s, 2H, NH, D_2O exchangeable), 7.66 (d, 2H, Ar-H), 7.84 (d, 2H, Ar-H); MS, m/z (%): 363 [M^+] (19) 280 [M^+ - C_6H_{11}] (89), 97 [$C_6H_{11}N$] (100); Analysis: calcd. for $C_{15}H_{21}N_7O_2S$ (363.44): C, 49.57; H, 5.82; N, 26.98; S, 8.82%; found: C, 49.40; H, 5.97; N, 27.12; S, 9.01%.

***N*-Butyl-4-[(3,5-diamino-1-methyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (5)**

A mixture of the hydrazone derivative **3b** (3.05 g, 0.01 mol) and methylhydrazine (0.54 mL, 0.01 mol) in absolute ethanol (20 mL) was refluxed for 4 h. The formed precipitate was filtered, dried and recrystallized from dioxane to give title compound **5**.

Yield 62%; m.p. 198-199°C; IR (KBr, cm^{-1}): 3390, 3225, 3149 (NH, $2NH_2$), 2927, 2847 (CH-aliph.), 1328, 1160 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.81 (t, 3H, CH_3), 1.26 (m, 2H, CH_2), 1.38 (m, 2H, CH_2), 2.59 (t, 2H, CH_2), 2.88 (s, 3H, -NCH_3), 6.20, 6.75 (2br s, 4H. $2NH_2$, D_2O exchangeable), 7.23 (br s, 1H, NH, D_2O exchangeable), 7.74 (d, 2H, Ar-H), 7.85 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.80 (CH_3), 19.06, 30.98 ($2CH_2$), 34.65 (N-CH_3), 42.52 (N-CH_2), 80.32, 127.32-145.78 (8 Ar-C), 157.23 (C=N); MS, m/z (%): 351 [M^+] (16), 76 [C_6H_4] (100); Analysis: calcd. for $C_{14}H_{21}N_7O_2S$ (351.43): C, 47.85; H, 6.02; N, 27.90; S, 9.12%; found: C, 47.85; H, 6.27; N, 27.78; S, 9.39%.

General procedure for the synthesis of *N*-substituted-4-[(3,5-diamino-1-phenyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamides 6a,b

A mixture of the hydrazone derivative **3b**,**c** (0.01 mol) and phenylhydrazine (1.08 mL, 0.01 mol) in absolute ethanol (20 mL) was refluxed for 3 h. The formed precipitate was filtered, dried and recrystallized from dioxane to give title compounds **6a**,**b**, respectively.

***N*-Butyl-4-[(3,5-diamino-1-phenyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (6a)**

Yield 51%; m.p. 180-181°C; IR (KBr, cm^{-1}): 3457, 3352, 3250 (NH, $2NH_2$), 2928, 2865 (CH-aliph.), 1345, 1152 (SO_2); ^1H NMR ($CDCl_3$, δ, ppm): 0.84 (t, 3H, CH_3), 1.29 (m, 2H, CH_2), 1.42 (m, 2H, CH_2), 2.96 (t, 2H, CH_2), 4.51, 5.07 (2br s, 4H, $2NH_2$, D_2O exchangeable), 6.61 (br. s, 1H, NH, D_2O exchangeable), 7.37-7.89 (m, 9H, Ar-H); ^{13}C NMR ($CDCl_3$, δ, ppm): 14.51 (CH_3), 20.21, 30.67 ($2CH_2$), 41.92 (N-CH_2), 86.72, 121.01-146.04 (14 Ar-C), 157.64 (C=N); MS, m/z (%): 413 [M^+] (12), 58 [C_3H_8N] (100). Analysis: calcd. for $C_{19}H_{23}N_7O_2S$ (413.50): C, 55.19; H, 5.61; N, 23.71; S, 7.75%; found: C, 54.98; H, 5.77; N, 23.84; S, 7.95%.

***N*-Cyclohexyl-4-[(3,5-diamino-1-phenyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (6b)**

Yield 74%; m.p. 238-239°C; IR (KBr, cm^{-1}): 3461, 3358, 3282 (NH, NH_2), 2928, 2861 (CH-aliph.), 1335, 1151 (SO_2); ^1H NMR ($CDCl_3$, δ, ppm): 1.21-1.77 (m, 10H, $5CH_2$- cyclohexyl protons), 2.49-2.64 (1H, m, -NCH- cyclohexyl proton), 4.56, 5.10 (2br s, 4H, $2NH_2$, D_2O exchangeable), 6.63 (br s, 1H, NH, D_2O exchangeable), 6.87-7.83 (m, 9H, Ar-H); MS, m/z (%): 439 [M^+] (25), 200 [M^+ - $C_{12}H_{16}NO_2S$] (18.4), 76 [C_6H_4] (100%); Analysis: calcd. for $C_{21}H_{25}N_7O_2S$(439.53): C, 57.38; H, 5.73; N, 22.31; S, 7.30%; found: C, 57.51; H, 5.94; N, 22.16; S, 7.47%.

***N*-Butyl-4-[(3,5-diamino-1-(2-cyanoacetyl)-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (7)**

A mixture of hydrazone **3b** (3.05 g, 0.01 mol) and 2-cyanoacetohydrazide (0.99 g, 0.01 mol) in absolute ethanol (20 mL) was refluxed for 6 h. The formed precipitate was filtered, dried and recrystallized from methanol to give title compound **7**.

Yield: 63%; m.p. 129-130°C; IR (KBr, cm^{-1}): 3407, 3308, 3220 (NH, $2NH_2$), 2934, 2862 (CH-aliph.), 2187 (CN), 1724 (CO), 1365, 1142 (SO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.83 (t, 3H, CH_3), 1.26 (m, 2H, CH_2), 1.43 (m, 2H, CH_2), 2.91 (t, 2H, CH_2), 3.70 (s, 2H, -$COCH_2CN$), 4.85, 6.47 (2br. s, 4H. $2NH_2$, D_2O exchangeable), 6.57 (br. s, 1H, NH, D_2O exchangeable), 7.57, 7.75 (2d, 4H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.25 (CH_3), 18.98, 20.54, 31.56 ($3CH_2$), 40.47 (N-CH_2), 116.32 (CN), 82.48, 126.56-148.21 (8 Ar-C), 154.30 (C=N), 193.67 (C=O); MS, m/z (%): 262 [M^+ - $C_7H_{14}N_2O$] (32), 233 [$C_{10}H_{13}N_6O$] (100); Analysis: calcd. for $C_{16}H_{20}N_8O_3S$ (404.45): C, 47.51; H, 4.98; N, 27.71; S, 7.93%; found: C, 47.81; H, 4.67; N, 27.45; S, 7.74%.

General procedure for the synthesis of *N*-substituted-4-[(3,5-diamino-1-(4-substitutedbenzoyl)-1*H*-pyrazol-4-yl]diazenyl)benzenesulfonamides 8a,b

A mixture of either hydrazone derivative **3b** (0.61 g, 0.002 mol) and *p*-chlorobenzoyl hydrazine (0.27 mL, 0.002 mol) or hydrazone derivative **3c** (0.67 g, 0.002 mol) and benzoyl hydrazine (0.34 g, 0.002 mol) in dioxane (20 mL) was refluxed for 12-16 h. After cooling, the reaction mixture was poured onto ice/cold water. The formed precipitate was filtered, dried and recrystallized from ethanol to give title compounds **8a**,**b**, respectively.

***N*-Butyl-4-[(3,5-diamino-1-(4-chlorobenzoyl)-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (8a)**

Yield 61%; m.p. 210-211°C; IR (KBr, cm^{-1}): 3420, 3294, 3213, 3170 (NH, NH_2), 2930, 2869 (CH-aliph.), 1686 (CO), 1360, 1153 (SO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.75 (t, 3H, CH_3), 1.20 (m, 2H, CH_2), 1.30 (m, 2H, CH_2), 2.71 (m, 2H, CH_2), 6.40, 8.56 (2br s, 4H. $2NH_2$, D_2O exchangeable), 7.56-7.98 (m, 8H, Ar-H), 10.63 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.41 (CH_3), 19.18, 31.03 ($2CH_2$), 42.19 (N-CH_2), 114.45-139.94 (14 Ar-C), 155.23 (C=N), 167.85 (C=O); MS, m/z (%): 477, 475 [M^+] (8, 25), 76 [C_6H_4] (100); Analysis: calcd. for $C_{20}H_{22}ClN_7O_3S$ (475.95): C, 50.47; H, 4.66; N, 20.60; S, 6.74%; found: C, 50.31; H, 4.78; N, 20.48; S, 6.89%.

***N*-Cyclohexyl-4-[(3,5-diamino-1-benzoyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (8b)**

Yield 55%; m.p. 171-173°C; IR (KBr, cm^{-1}): 3390, 3383, 3171 (NH, $2NH_2$), 2928, 2861 (CH-aliph.), 1678 (CO), 1362, 1149 (SO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.23-1.72 (m, 10H, $5CH_2$-cyclohexyl protons), 2.52-2.68 (1H, m, -NCH-cyclohexyl proton), 6.35, 8.51 (2br s, 4H, $2NH_2$, D_2O exchangeable), 7.37-7.75 (m, 9H, Ar-H), 10.43 (br s, 1H, NH, D_2O exchangeable); MS, m/z (%): 467 [M^+] (27), 105 [C_7H_5O] (100), 77 [C_6H_5] (50). Analysis: calcd. for $C_{22}H_{25}N_7O_3S$ (467.54): C, 56.52; H, 5.39; N, 20.97; S, 6.86%; found: C, 56.36; H, 5.57; N, 20.79; S, 7.01%.

General procedure for the synthesis of *N*-substituted-4-[(3,5-diamino-1- substituted thiocarbamoyl-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamides 9a-c

A mixture of hydrazone derivative **3b,c** (0.01 mol), the appropriate thiosemicarbazide derivatives namely: ethylthiosemicarbazide and/or phenylthiosemicarbazide or thiosemicarbazide (0.01 mol) and triethylamine (0.5 mL) in dioxane (20 mL) was refluxed for 10-12 h. The formed precipitate was filtered, dried and recrystallized from ethanol to give title compounds **9a**-**c**, respectively.

***N*-Butyl-4-[(3,5-diamino-1-(*N*-ethylthiocarbamoyl)-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (9a)**

Yield 57%; m.p. >300°C; IR (KBr, cm^{-1}): 3427, 3321, 3277 (2NH, $2NH_2$), 2931, 2869 (CH-aliph.), 1352, 1146 (SO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (m, 6H, CH_3), 1.24 (m, 2H, CH_2), 1.33 (t, 3H, -NCH_2CH_3), 1.45 (m, 2H, CH_2), 1.79 (q, 2H, -NCH_2CH_3), 2.97 (t, 2H, CH_2), 3.67, 4.37 (2br s, 4H. $2NH_2$, D_2O exchangeable), 3.33, 8.50 (2br s, 2H. 2NH, D_2O exchangeable), 7.84, 7.96 (2d, 4H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.23, 14.98 ($2CH_3$), 19.25, 31.35, 34.65 ($3CH_2$), 42.87 (N-CH_2), 87.23, 125.98-141.26 (8 Ar-C), 152.20 (C=N), 189.03 (C=S); MS, m/z (%): 424 [M^+] (14), 336 [M^+ - C_3H_6NS] (13), 233 [$C_9H_9N_6S$] (100); Analysis: calcd. for $C_{16}H_{24}N_8O_2S_2$ (424.54): C, 45.27; H, 5.70; N, 26.39; S, 15.11%; found: C, 45.12; H, 5.45; N, 26.49; S, 14.98%.

***N*-Butyl-4-[(3,5-diamino-1-(*N*-phenylthiocarbamoyl)-1*H*-pyrazol-4-yl)diazenyl]benzenesulfonamide (9b)**

Yield 52%; m.p. 292-293°C; IR (KBr, cm^{-1}): 3403, 3384, 3365, 3237 (2NH, $2NH_2$), 2926, 2864 (CH-aliph.), 1356, 1145 (SO_2); ^{1}H NMR (DMSO-d_6, δ, ppm): 0.86 (t, 3H, CH_3), 1.13 (m, 2H, CH_2), 1.27 (m, 2H, CH_2), 2.46 (m, 2H, CH_2), 6.38, 7.96 (2br s,

4H. $2NH_2$, D_2O exchangeable), 7.34-7.85 (m, 10H, Ar-H, NH, D_2O exchangeable), 9.87 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.91 (CH_3), 20.56, 30.28 ($2CH_2$), 42.55 (N-CH_2), 86.27, 126.56-140.10 (14 Ar-C), 152.24 (C=N), 187.01 (C=S); MS, m/z (%): 472 [M^+] (62), 93[C_6H_7N] (100); Analysis: calcd. for $C_{20}H_{24}N_8O_2S_2$ (472.59): C, 50.83; H, 5.12; N, 23.71; S, 13.57%; found: C, 50.68; H, 5.05; N, 23.56; S, 13.62%.

_N_-Cyclohexyl-4-[(3,5-diamino-1-thiocarbamoyl-1_H_-pyrazol-4-yl)diazenyl]benzenesulfonamide (9c)

Yield 46%; m.p. 161-163°C; IR (KBr, cm^{-1}): 3429, 3398, 3359, 3246 (NH, NH_2), 2922 (CH-aliph.), 1350, 1140 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 1.19-1.59 (m, 10H, $5CH_2$- cyclohexyl protons), 2.35-2.43 (m, 1H, -NCH- cyclohexyl proton), 5.12, 5.97 (2br. s, 6H, $3NH_2$, D_2O exchangeable); 7.29 (d, 2H, Ar-H), 7.77 (d, 2H, Ar-H), 11.14 (br s, 1H, NH, D_2O); MS, m/z (%): 422 [M^+] (29), 346 [M^+ - CH_4N_2S] (43), 139 [$C_6H_3O_2S$] (100); Analysis: calcd. for $C_{16}H_{22}N_8O_2S_2$(422.53): C, 45.48; H, 5.25; N, 26.52; S, 15.18%; found: C, 45.32; H, 5.15; N, 26.41; S, 15.29%.

4-[(4-Amino-2-(((4-(_N_-butylsulfamoyl)phenyl)diazenyl)(cyano)methyl)-6-(naphthalen-1-yloxy)pyrimidin-5-yl)diazenyl]-_N_-butylbenzenesulfonamide (10)

A mixture of hydrazone derivatives **3b** (1.22 g, 0.004 mol) and á-naphthol (0.29 g, 0.002 mol) in absolute ethanol (20 mL) was refluxed for 4 h. After cooling the formed precipitate was filtered, dried and recrystallized from benzene to give title compound **10**.

Yield 65%; m.p. 123-124°C; IR (KBr, cm^{-1}): 3431, 3312, 3201 (2NH, NH_2), 2938, 2866 (CH-aliph.), 2189 (CN), 1356, 1138 (SO_2); ^1H NMR ($CDCl_3$, δ, ppm): 0.81-0.84 (m, 6H, $2CH_3$), 1.26-1.29 (m, 4H, $2CH_2$), 1.41-1.48 (m, 4H, $2CH_2$), 2.89-2.95 (m, 4H, $2CH_2$), 3.69 (s, 1H, -CH-CN), 4.91 (br s, 2H, NH_2, D_2O exchangeable), 7.25-7.78 (m, 15H, Ar-H) 6.61, 8.20 (2br s, 2H, 2NH, D_2O exchangeable); ^{13}C NMR ($CDCl_3$, δ, ppm): 13.64 ($2CH_3$), 19.98, 31.26 ($4CH_2$), 42.04 (2N-CH_2), 54.65 (CH), 118.90 (CN), 120.65-149.83 (24 Ar-C), 153.12, 153.98 (2C=N); MS, m/z (%): 738 [M^+ - NH_3] (11), 233 [$C_{14}H_7N_3O$] (100). Analysis: calcd. for $C_{36}H_{38}N_{10}O_5S_2$ (754.88): C, 57.28; H, 5.07; N, 18.55; S, 8.50%; found: C, 57.09; H, 5.22; N, 18.41; S, 8.65%

General procedure for the synthesis of _N_-butyl-4-[(4,6-diamino-2-(oxo or thioxo)-2,5-dihydropyrimidin-5-yl)diazenyl]benzenesulfonamide 11a,b

A mixture of hydrazone derivative **3b** (3.05 g, 0.01 mol) urea and/or thiourea (0.01 mol) in ethanolic sodium hydroxide (10%, 20 mL) was refluxed for 6-10 h. The reaction mixture was cooled, poured onto ice/cold water and acidified by diluted hydrochloric acid. The formed precipitate was filtered, washed several times with water, dried and recrystallized from ethanol to give title compounds **11a**,**b**, respectively.

_N_-Butyl-4-[(4,6-diamino-2-oxo-2,5-dihydropyrimidin-5-yl)diazenyl]benzenesulfonamide (11a)

Yield 41%; m.p. 231-233°C; IR (KBr, cm^{-1}): 3398, 3386, 3343, 3294 (NH, $2NH_2$), 2926 (CH-aliph.), 1687 (C=O), 1358, 1139 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.76 (t, 3H, CH_3), 1.22 (m, 2H, CH_2), 1.28 (m, 2H, CH_2), 2.65 (m, 2H, CH_2), 4.30 (s, 1H, CH-pyrimidine), 5.89, 6.03 (2br s, 4H, $2NH_2$, D_2O exchangeable), 7.70 (d, 2H, Ar-H), 7.77 (d, 2H, Ar-H), 8.21 (br s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ, ppm): 14.02 (CH_3), 19.87, 32.21 ($2CH_2$), 41.65 (N-CH_2), 54.32 (CH), 128.21-140.92 (6 Ar-C), 153.34 (2C=N), 169.96 (C=O); MS, m/z (%): 365 [M^+] (16), 241 [$C_{10}H_{15}N_3O_2S$] (22), 76 [C_6H_4] (100); Analysis: calcd. for $C_{14}H_{19}N_7O_3S$ (365.41): C, 46.02; H, 5.24; N, 26.83; S, 8.78%; found: C, 45.87; H, 5.42; N, 26.99; S, 8.65%.

_N_-Butyl-4-[(4,6-diamino-2-thioxo-2,5-dihydropyrimidin-5-yl]diazenyl)benzenesulfonamide (11b)

Yield 56%; m.p. > 300°C; IR (KBr, cm^{-1}): 3405, 3396, 3341, 3298 (NH, $2NH_2$), 2934 (CH-aliph.), 1351, 1143 (SO_2), 1057 (CS); ^1H NMR (DMSO-d_6, δ, ppm): 0.71 (t, 3H, CH_3), 1.19 (m, 2H, CH_2), 1.32 (m, 2H, CH_2), 2.69 (m, 2H, CH_2), 4.35 (s, 1H, CH- pyrimidine), 5.84, 6.15 (2br s, 4H, $2NH_2$, D_2O exchangeable), 7.65 (d, 2H, Ar-H), 7.79 (d, 2H, Ar-H), 8.03 (br s, 1H, NH, D_2O exchangeable); MS, m/z (%): 381 [M^+] (21), 351 [M^+ - C_2H_6] (7), 241 [$C_{10}H_{15}N_3O_2S$] (9), 233 [$C_{10}H_{11}N_5S$] (100); Analysis: calcd. for $C_{14}H_{19}N_7O_2S_2$ (381.48): C, 44.08; H, 5.02; N, 25.70; S, 16.81%; found: C, 44.17; H, 5.25; N, 25.59; S, 16.98%.

4-[(3,5-diaminoisoxazol-4-yl)diazenyl]-_N_-butylbenzenesulfonamide (12)

A mixture of hydrazone derivative **3b** (3.05 g, 0.01 mol) and hydroxylamine hydrochloride (1.04 g, 0.015 mol) in methanolic sodium hydroxide (10%, 20 mL) was refluxed for 10 h. The reaction mixture was cooled, poured onto ice/cold water acidified by diluted hydrochloric acid. The formed precipitate was filtered, washed several times with water, dried

and recrystallized from methanol to give the title compound **12**.

Yield 43%; m.p. 123-124°C; IR (KBr, cm^{-1}): 3438, 3382, 3268 (NH, $2NH_2$), 2929, 2868 (CH-aliph.), 1322, 1155 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.77 (t, 3H, CH_3), 1.19 (m, 2H, CH_2), 1.31 (m, 2H, CH_2), 2.72 (m, 2H, CH_2), 6.04, 6.78, 11.97 (3br s, 5H, $2NH_2$, NH, D_2O exchangeable), 7.68 (d, 2H, Ar-H), 7.89 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 14.23 (CH_3), 21.05, 31.84 ($2CH_2$), 42.63 (N-CH_2), 99.71, 126.59-138.14 (8 Ar-C), 156.04 (C=N); MS, m/z (%): 338 [M^+] (70), 266 [M^+ - $C_4H_{10}N$] (94), 202 [M^+-$C_4H_{10}NO_2S$] (100); Analysis: calcd. for $C_{13}H_{18}N_6O_3S$ (338.39): C, 46.14; H, 5.36; N, 24.84; S, 9.48%; found: C, 46.03; H, 5.21; N, 25.01; S, 9.56%.

General procedure for the synthesis of 4-[(2-amino-1-cyano-2-(substituted-1-yl)vinyl) diazenyl]-*N*-butyl-benzenesulfonamides 13a-c

A mixture of hydrazone derivative **3b** (0.61 g, 0.002 mol) and the appropriate secondary amine namely: piperidine, 2-methylpiperidine and/or 1-methylpiperazine (0.002 mol) in dioxane (15 mL) was refluxed for 4-10 h. The reaction mixture was cooled, poured onto ice/cold water acidified by diluted hydrochloric acid. The formed precipitate was filtered, dried and recrystallized from ethanol to give title compounds **13a-c**, respectively.

4-[(2-Amino-1-cyano-2-(piperidin-1-yl)vinyl) diazenyl]-*N*-butylbenzenesulfonamide (13a)

Yield 73%; m.p. 102-103°C; IR (KBr, cm^{-1}): 3403, 3306, 3285 (NH, NH_2), 2936, 2864 (CH-aliph.), 2187 (CN), 1322, 1152 (SO_2); ^1H NMR ($CDCl_3$, δ, ppm): 0.82 (t, 3H, CH_3), 1.27 (m, 2H, CH_2), 1.42 (m, 2H, CH_2), 1.62-1.75 (m, 6H, $3CH_2$- piperidine protons), 2.89 (t, 2H, CH_2), 3.68 (m, 4H, $2CH_2$- piperidine protons), 6.35, 6.67 (2br s, 3H, NH_2, NH, D_2O exchangeable) 7.61 (d, 2H, Ar-H), 7.75 (d, 2H, Ar-H); ^{13}C NMR ($CDCl_3$, δ, ppm): 13.93 (CH_3), 19.74, 24.32, 26.75, 31.29 ($5CH_2$), 42.46, 49.31 (3N-CH_2), 118.98 (CN), 122.01-140.17 (8 Ar-C); MS, m/z (%): 390 [M^+] (10), 156 [$C_8H_{18}N_3$] (53), 85 [$C_5H_{11}N$] (100); Analysis: calcd. for $C_{18}H_{26}N_6O_2S$ (390.50): C, 55.36; H, 6.71; N, 21.52; S, 8.21%; found: C, 55.58; H, 6.93; N, 21.36; S, 8.04%.

4-[(2-Amino-1-cyano-2-(2-methylpiperidin-1-yl)vinyl)diazenyl]-*N*-butylbenzenesulfonamide (13b)

Yield 58%; m.p. 97-99°C; IR (KBr, cm^{-1}): 3412, 3394, 3385 (NH, NH_2), 2932, 2860 (CH-aliph.), 2226 (CN), 1330, 1148 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.85 (t, 3H, CH_3), 1.11 (m, 3H, CH_3), 1.26 (m, 2H, CH_2), 1.40 (m, 2H, CH_2), 1.59, 1.73 (m, 6H, $3CH_2$- piperidine protons), 2.86 (t, 2H, CH_2), 3.64 (m, 3H, piperidine protons), 6.30, 11.58 (2br s, 3H, NH_2, NH, D_2O exchangeable) 7.56 (d, 2H, Ar-H), 7.68 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 14.65, 16.69 ($2CH_3$), 20.32, 23.94, 27.24, 31.01 ($5CH_2$), 42.31, 43.84 (2N-CH_2), 53.02 (CH), 119.05 (CN), 121.45-139.32 (8 Ar-C); MS, m/z (%): 404 [M^+] (34), 202 [$C_9H_7N_4S$] (100); Analysis: calcd. for $C_{19}H_{28}N_6O_2S$ (404.53): C, 56.41; H, 6.98; N, 20.77; S, 7.93%; found: C, 56.23; H, 7.18; N, 21.05; S, 8.16%.

4-[(2-Amino-1-cyano-2-(4-methylpiperazin-1-yl)vinyl)diazenyl]-*N*-butylbenzenesulfonamide (13c)

Yield 39%, m.p. 68-69°C; IR (KBr, cm^{-1}): 3394, 3371, 3287 (NH, NH_2), 2917, 2857 (CH-aliph.), 2223 (CN), 1331, 1153 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.91 (t, 3H, CH_3), 1.27 (m, 2H, CH_2), 1.39 (m, 2H, CH_2), 2.69 (t, 2H, CH_2), 2.71 (s, 3H, -NCH_3), 2.81 (m, 4H, $2CH_2$- piperazine protons), 3.51 (m, 4H, $2CH_2$- piperazine protons), 6.42, 11.21 (2br s, 3H, NH_2, NH, D_2O exchangeable) 7.61 (d, 2H, Ar-H), 7.71 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 14.03 (CH_3), 19.30, 32.64 ($2CH_2$), 38.84 (N-CH_3), 42.03, 49.80, 54.02 (5N-CH_2), 118.35 (CN), 123.14-142.65 (8 Ar-C); MS, m/z (%): 405 [M^+] (18), 365 [$C_{17}H_{27}N_5O_2S$] (65), 76 [C_6H_4] (100); Analysis: calcd. for $C_{18}H_{27}N_7O_2S$ (405.52): C, 53.30; H, 6.71; N, 24.18; S, 7.91%; found: C, 53.16; H, 6.93; N, 24.03; S, 7.84%.

General procedure for the synthesis of *N*-butyl-4-[2-(5,7-diamino-2,3-dihydro-6*H*-1,4-diazepin-6-ylidene)hydrazinyl]benzenesulfonamide (14), *N*-butyl-4-[2-(2,4-diamino-3*H*-benzo[b][1,4] diazepin-3-ylidene)hydrazinyl]benzenesulfonamide (15) and *N*-butyl-4-[2-(4-amino-2-iminobenzo[b][1,4]oxazepin-3(2*H*)-ylidene)hydrazinyl]benzenesulfonamide (16)

A mixture of hydrazone derivative **3b** (0.61 g, 0.002 mol) and the appropriate diamine namely: 1,2-diaminoethane, *O*-phenylenediamine and/or *O*-aminophenol (0.002 mol) in absolute ethanol (15 mL) containing a catalytic amount of piperidine (0.2 mL) was refluxed for 5 h. After cooling, the formed precipitate was filtered, dried and recrystallized from ethanol to give title compounds **14-16,** respectively.

***N*-butyl-4-[2-(5,7-diamino-2,3-dihydro-6*H*-1,4-diazepin-6-ylidene)hydrazinyl]benzenesulfonamide (14)**

Yield 44%; m.p. 134-135°C; IR (KBr, cm^{-1}): 3415, 3389, 3376, 3268 (NH, $2NH_2$), 2954, 2933, 2865 (CH-aliph.), 1580 (C=N), 1313, 1141 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.79 (t, 3H, CH_3), 1.16-1.38 (m, 8H, $2CH_2$- butyl protons, $2CH_2$- diazepine protons), 1.65 (br s, 4H, $2NH_2$, D_2O exchangeable), 2.71 (t, 2H, CH_2), 7.55-7.80 (m, 4H, Ar-H, NH, D_2O exchangeable); MS, m/z (%): 365 [M^+] (59), 366 [M^++1] (56), 54 [C_4H_6] (100). Analysis: calcd. for $C_{15}H_{23}N_7O_2S$ (365.45): C, 49.30; H, 6.34; N, 26.83; S, 8.77%; found: C, 49.13; H, 6.23; N, 27.01; S, 8.89%.

_N_-butyl-4-[2-(2,4-diamino-3_H_-benzo[b][1,4] diazepin-3-ylidene)hydrazinyl]benzenesulfonamide (15)

Yield 52%; m.p. 103-104°C; IR (KBr, cm^{-1}): 3433, 3395, 3380, 3265 (NH, $2NH_2$), 2939, 2860 (CH-aliph.), 1607 (C=N), 1320, 1146 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.79 (t, 3H, CH_3), 1.24 (m, 2H, CH_2), 1.34 (m, 2H, CH_2), 1.66 (br s, 4H, $2NH_2$, D_2O exchangeable), 2.73 (t, 2H, CH_2), 7.36-7.97 (m, 9H, Ar-H, NH, D_2O exchangeable); MS, m/z (%): 413 [M^+] (74), 412 [M^+-1] (92), 371 [M^+ - C_3H_6] (100). Analysis: calcd. for $C_{19}H_{23}N_7O_2S$ (413.50): C, 55.19; H, 5.61; N, 23.71; S, 7.75%; found: C, 54.96; H, 5.42; N, 23.85; S, 7.89%.

_N_-butyl-4-[2-(4-amino-2-iminobenzo[b][1,4] oxazepin-3(2_H_)-ylidene)hydrazinyl]benzenesulfonamide (16)

Yield 57%; m.p. 119-120°C; IR (KBr, cm^{-1}): 3397, 3311, 3282 (2NH, NH_2), 2937, 2866 (CH-aliph.), 1616 (C=N), 1321, 1143 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.78 (t, 3H, CH_3), 1.21 (m, 2H, CH_2), 1.33 (m, 2H, CH_2), 1.65 (br s, 2H, NH_2, D_2O exchangeable), 2.72 (t, 2H, CH_2), 4.32 (br s, 2H, 2NH, D_2O exchangeable), 7.18-7.81 (m, 9H, Ar-H, NH, D_2O exchangeable); MS, m/z (%): 414 [M^+] (62), 415 [M^++1] (90), 308 [M^+ - $C_4H_{12}NO_2$] (100); Analysis: calcd. for $C_{19}H_{22}N_6O_3S$ (414.48): C, 55.06; H, 5.35; N, 20.28; S, 7.74%; found: C, 54.96; H, 5.42; N, 20.56; S, 7.89%.

_N_-butyl-4-(6,8-diamino-4,7-dicyano-3-iminopyrido[3,2-c]pyridazin-2(3_H_)-yl)benzene sulfonamide (18)

Method A

A mixture of hydrazone derivative **3b** (3.05 g, 0.01 mol) and malononitrile (1.32 g, 0.02 mol) in absolute ethanol (20 mL) containing a catalytic amount of triethylamine (1 mL) was refluxed for 12 h. The formed precipitate was filtered, dried and recrystallized from DMF/ethanol (1 : 2, v/v) to give title compound **18**.

Method B

A mixture of compound **20** (3.71 g, 0.01 mol) and malononitrile (0.66 g, 0.01 mol) in absolute ethanol (25 mL) containing a catalytic amount of triethylamine (1 mL) was refluxed for 6 h. The formed precipitate was filtered, dried and recrystallized from DMF/ethanol (1 : 2, v/v) to give title compound **18**.

Yield 63%; m.p.174-175°C; IR (KBr, cm^{-1}): broad band centered at 3330 (2NH, $2NH_2$), 2930, 2870 (CH-aliph.), 2205 (CN), 1382, 1152 (SO_2); ^1H NMR (DMSO-d_6, δ, ppm): 0.75 (t, 3H, CH_3), 1.18 (m, 2H, CH_2), 1.28 (m, 2H, CH_2), 2.72 (t, 2H, CH_2), 7.76 (d, 2H, Ar-H), 8.10 (d, 2H, Ar-H), 4.26, 8.11, 9.19, 9.88 (4br s, 6H, 2NH + $2NH_2$, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, δ, ppm): 13.89 (CH_3), 20.03, 31.64 ($2CH_2$), 42.94 (N-CH_2), 119.67 (2CN), 122.34-146.34 (11 Ar-C), 156.32, 157.19 (2C=N); MS, m/z (%): 437 [M^+] (18), 365 [$M^+ - C_4H_{10}N$] (5), 139 [C_6H_5NOS] (100); Analysis: calcd. for $C_{19}H_{19}N_9O_2S$ (437.48): C, 52.16; H, 4.38; N, 28.82; S, 7.33%; found: C, 52.02; H, 4.58; N, 29.01; S, 7.50%.

2-Amino-_N'_-(4-(N-butylsulfamoyl)phenyl)-3,3-dicyanoacrylohydrazonoyl cyanide (20)

To a cold mixture of sodium acetate (3.0 g) and malononitrile dimer (MND) **19** (0.01 mol) in ethanol (30 mL), a solution of diazonium salt **2b** was added and stirred for 5 h. The resulting solid was washed with water (100 mL) and recrystallized from dioxane to give the title compound **20**.

Yield 42%; m.p. > 300 °C; IR (KBr, cm^{-1}): 3410, 3365, 3261 (NH, NH_2), 2930, 2856 (CH-aliph.), 2219, 2229 (CN); ^1H NMR (DMSO-d_6, δ, ppm): 0.75 (t, 3H, CH_3), 1.18 (m, 2H, CH_2), 1.29 (m, 2H, CH_2), 2.63 (m, 2H, CH_2), 6.14, 8.45, 10.30 (3br s, 4H, 2NH, NH_2, D_2O exchangeable), 7.63 (d, 2H, Ar-H), 7.75 (d, 2H, Ar-H); ^{13}C NMR (DMSO-d_6, δ, ppm): 14.25 (CH_3), 21.24, 31.10 ($2CH_2$), 42.36 (N-CH_2), 117.32, 119.02 (3CN), 69.89, 120.32-147.46 (8 Ar-C), 156.79 (C=N); MS, m/z (%): 371 [M^+] (13), 76 [C_6H_4] (100); Analysis: calcd. for $C_{16}H_{17}N_7O_2S$ (371.42): C, 51.74; H, 4.61; N, 26.40; S, 8.63%; found: C, 51.89; H, 4.42; N, 26.58; S, 8.89%.

Biological screening

Anti-inflammatory assay

Male Wistar rats weighing 120-150 g were used throughout the assay. Animals were housed under standardized conditions for light and temperature and received standard rat chow and tap water *ad libitum*. Animals were randomly assigned to dif-

ferent experimental groups, each of six rats and kept in separate cages. One group of six rats was kept as a control and another group received the standard drug celecoxib. All animal procedures were performed after approval from the Ethics Committee of the National Research Centre and in accordance with the recommendations for the proper care and use of laboratory animals (NIH publication No. 85-23, revised 1985).

Carrageenan lambda from Sigma Aldrich Chemical Co. (USA), celecoxib from Pfizer Co. (Egypt).

Paw edema was induced by subplantar injection of 100 µL of 1% sterile carrageenan in saline into the right hind paw (1% suspension of carrageenan in sterile saline was prepared, the suspension was placed in a refrigerator (4°C) overnight to allow complete hydration of the carrageenan (30).

Sixteen groups of rats, each of six animals, were used. One group received distilled water and served as a control; the tested compounds were administered orally at an equimolar oral dose relative to 50 mg/kg of celecoxib. The tested compounds were administered in a dose of 100 mg/kg orally and celecoxib in a dose of 50 mg/kg. All the tested compounds and celecoxib were orally administered one hour before induction of inflammation.

The hind paw volume was measured immediately before carrageenan injection and at selected times (1, 2, 3 and 4 h) thereafter by water displacement method using 7410, Ugo Basile, plythesmometer, Comerio, Italy (31). The apparatus works measuring the volume of an electrolyte solution displaced by the animal paw. The increased water level in the cell compartment increases the immersed portion of two parallel platinum electrodes, thus increasing conductivity between the two electrodes. The increased conductivity is converted to volume by a transducer (7153 Conductance Transducer).

The percent edema inhibition was calculated from the mean effect in the control and treated animals according to the following equation:

$$\% \text{ edema inhibition} = (vt - vc) / vc \times 100$$

where v*t* represents the mean increase in paw volume in rats treated with tested compounds and v*c* represents the mean increase in paw volume in the control group of rats.

Analgesic assay

Antinociceptive responses were determined using the tail-flick test (32, 33). To measure the latency of the tail-flick response, rats were gently held with the tail put on the apparatus (Ugo Basile, USA) for radiant heat stimuli. The tail flick response was elicited by applying radiant heat to the dorsal surface of the mouse-tail. The time in seconds, from initial heat source activation until tail withdrawal was recorded. The mean of two measures was used for each experimental animal as the tail withdrawal latency. In order to avoid excessive suffering of animals, a cut-off was set at 30 s reaction time.

Ulcerogenic assay

Groups of male Wistar rats with a weight between 120 and 150 g were used. They were starved 18 h prior to drug administration. The test compounds were administered orally in 100 mg/kg as aqueous suspension. The animals were sacrificed after 5 h. Stomachs were removed and examined. A longitudinal incision along the greater curvature was made with fine scissor. The presence of a single or multiple lesions (erosion, ulcer or perforation) was evaluated (34). The number of ulcers and the occurrence of hyperemia were noted.

Molecular docking

All docking studies were performed using Internal Coordinate Mechanics (Molsoft ICM 3.5-0a).

Preparation of small molecule

A set of new derivatives synthesized (**1b**, **3b**, **4a**, **5**, **6a**, **7**, **8a**, **8b**, **9b**, **10**, **11a**, **14**, **16**, **18**) were compiled by us using ChemDraw. 3D structures were constructed using Chem 3D ultra 12.0 software [Molecular Modeling and Analysis; Cambridge Soft Corporation, USA (2010)], then they were energetically minimized by using MOPAC (semi-empirical quantum mechanics), Job Type with 100 iterations and minimum RMS gradient of 0.01, and saved as MDL MolFile (* .mol).

Generation of ligand and enzyme structures

The crystal structure of target enzyme COX-2 (pdb code = 3LN1) was retrieved from the Protein Data Bank (http://www.rcsb.org/pdb/welcome.do). All bound water ligands and cofactors were removed from the protein.

Docking using Molsoft ICM 3.5-a. software

Convertion of our PDB file into an ICM object

This conversion involves addition of hydrogen bonds, assignment of atoms types, and charges from the residue templates.

To perform ICM small molecule docking the following procedure was used

a) Setup docking project:

1) Set project name

2) Setup the receptor
3) Review and adjust binding site
4) Make receptor maps:
b) Start docking simulation:
Display the result

ICM stochastic global optimization algorithm attempts to find the global minimum of the energy function that include five grid potentials describing interaction of the flexible ligand with the receptor and internal conformational energy of the ligand; during this process a stack of alternative low energy conformations is saved. All inhibitors were compared according to the best binding free energy (minimum) obtained among all runs.

RESULTS AND DISCUSSION

Chemistry

The synthesis of the designed target compounds were achieved as outlined in (Schemes 1-4). The synthestic route started with diazotization of sulfanilamide derivatives **1a-c** followed by coupling with malononitrile in a basic medium using one equivalent of sodium acetate to yield the corresponding intermediate hydrazones **3a-c**. (Scheme 1)

It was reported that various β-enaminonitriles reacted with different hydrazines to afford the corresponding pyrazole derivatives (35). Thus, the treatment of the compounds **3b**,**c** with hydrazine hydrate in ethanol yielded the corresponding 3,5-diaminopyrazole derivatives **4a**,**b**. Fruthermore, condensation of hydrazone **3b** with methyl hydrazine and hydrazones **3b**,**c** with phenyl hydrazine yielded the corresponding *N*-substituted diaminopyrazoles **5** and **6a**,**b**, respectively.

Also, condensation of hydrazone **3b** with cyanoacetohydrazide afforded the cyanoacetyl pyrazole derivative **7**. Additionally, refluxing the hydrazone **3b** with p-chlorobenzoyl hydrazine (29) and hydrazone **3c** with benzoylhydrazine in dioxane furnished the corresponding *N*-benzoyldiaminopyrazole derivatives **8a**,**b**. 3,5-Diamino-1-(*N*-substituted)thiocarbamoyl-4-[4-(*N*-substituted sulfamoyl)] phenylazopyrazoles **9a-c** were prepared *via* the condensation of hydrazones **3b**,**c** with N^4-substituted thiosemicarbazides namely; ethylthiosemicarbazide, phenylthiosemicarbazide and/or with thiosemicarbazide (Scheme 2).

This study was extended to investigate the behavior of the hydrazone derivative **3b** towards nucleophilic agent (36) such as α-naphthol to provide product **10** with the analytical and spectral data showing that two moles of the hydrazone derivative **3b** were consumed (37). Also, the pyrimidine derivatives **11a**,**b** were synthesized *via* the condensation of hydrazone derivative **3b** with urea and/or thiourea in ethanolic sodium hydroxide. Condensation of hydrazone derivative **3b** with hydroxylamine hydrochloride in ethanolic sodium hydroxide afforded 4-[(3-amino-5-imino-4,5-dihydroisoxazol-4-yl)diazenyl]-*N*-butylbenzenesulfonamide (**12**).

Furthermore, the reaction of hydrazone derivative **3b** with different secondary amines namely: piperidine, 2-methylpiperidine and/or 1-methylpiperazine in boiling ethanol produced enaminonitrile derivatives **13a-c**, respectively. Also, the hydrazone derivative **3b** was allowed to react with 1,2 diaminoethane, *O*-phenylenediamine and/or *O*-aminophenol in boiling ethanol containing a catalytic amount of piperidine to yield the corresponding 2,3-dihydro-6*H*-1,4-diazepine **14**, benzo[b][1,4]diazepine **15** and benzo[b][1,4]oxazepine **16**, respectively. (Scheme 3).

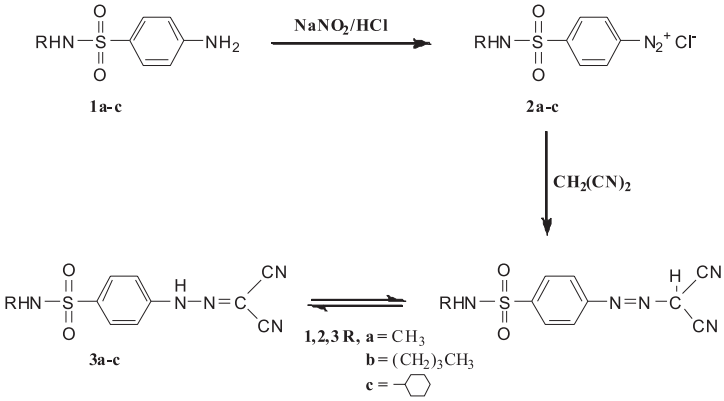
Moreover, a bicyclic nitrogen-containing heterocyclic compound such as pyrido-pyridazine derivative **18** was obtaind by two routes (38). First, by the reaction of the hydrazone derivative **3b** with two equivalents of malononitrile. Second, by coupling of the diazonium chloride salt **2b** with malononitrile dimer (MND) **19** followed by further cyclocondensation with another mole of malononitrile to obtain the desired product **18**. (Scheme 4)

Pharmacological screening

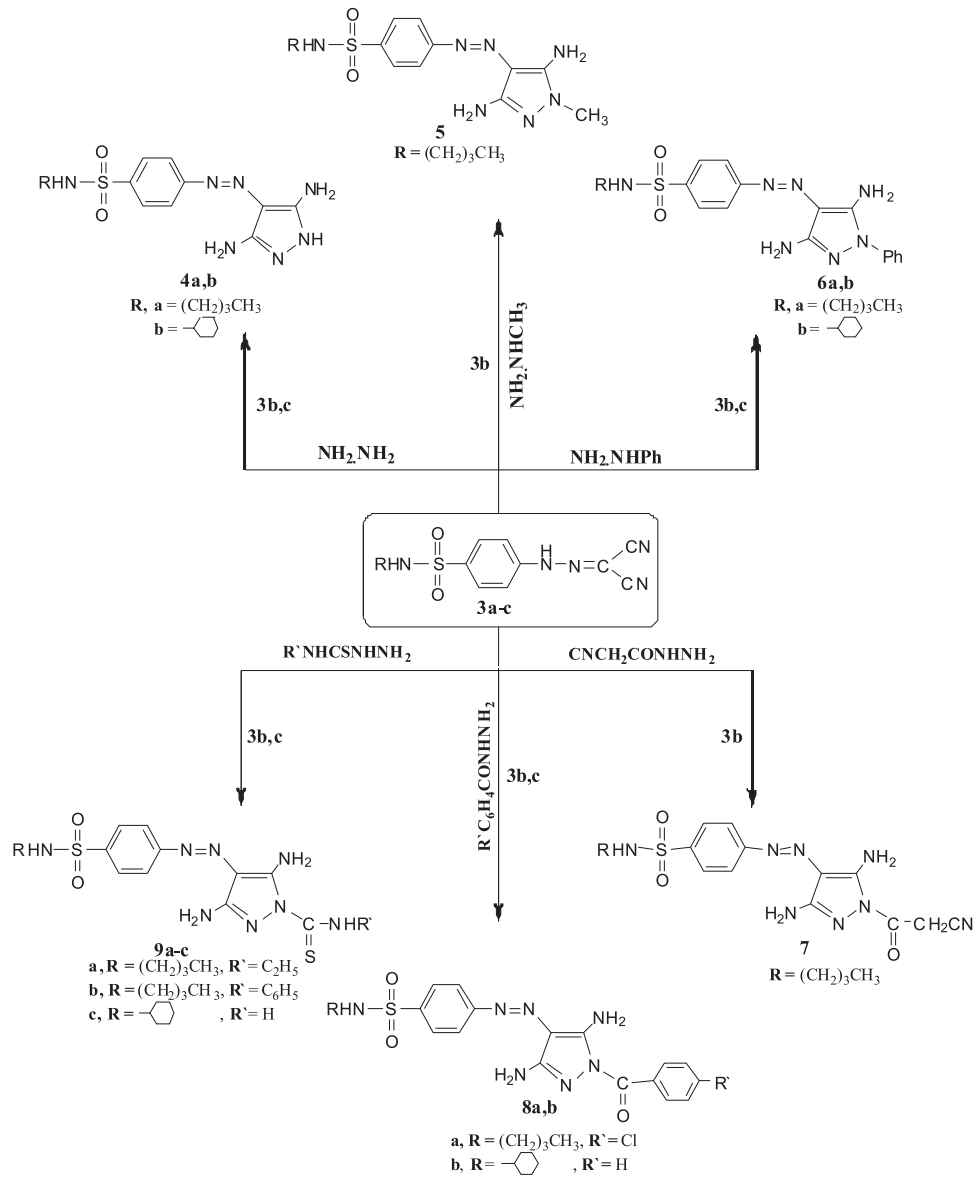
Anti-inflammatory activity

In this study, fourteen derivatives of the newly synthesized compounds (**1b**, **3b**, **4a**, **5**, **6a**, **7**, **8a**, **8b**, **9b**, **10**, **11a**, **14**, **16**, **18**) were selected as representative examples to evaluate their anti-inflammatory, analgesic and ulcerogenic activities.

As seen in Tables 1 and 2, the anti-inflammatory activity varies with variation of the heterocyclic rings attached to the parent azo-benzenesulfonamide nucleus. The highest anti-inflammatory activity, equipotent to that of the reference drug celecoxib, was gained by the starting azo-benzenesulfonamide derivative **1b**, carbonohydrazonoyl dicyanide **3b** and its cyclized 3,5-diaminopyrazole derivatives **4a**, **5** and benzo[1,4]oxazepine derivative **16**. They produced rapid anti-inflammatory action with long duration of action thus, the percentage of edema inhibition greatly increased up to the 4th h post their administration with values: -66.13, -77.12, -79.51 -67.44, -67.31, celecoxib; -76.33). It was also noticed that the conjugation of the pyrazole ring with phenyl, cyanoacetyl, benzoyl and/or phenylthiocarbamoyl moieties **6a**, **7**, **8a**, **9b** led to loss of their anti-inflammatory activity on 1st h post their administration, but activity moderately increased on the



Scheme 1. Synthesis of target compounds **3a-c**



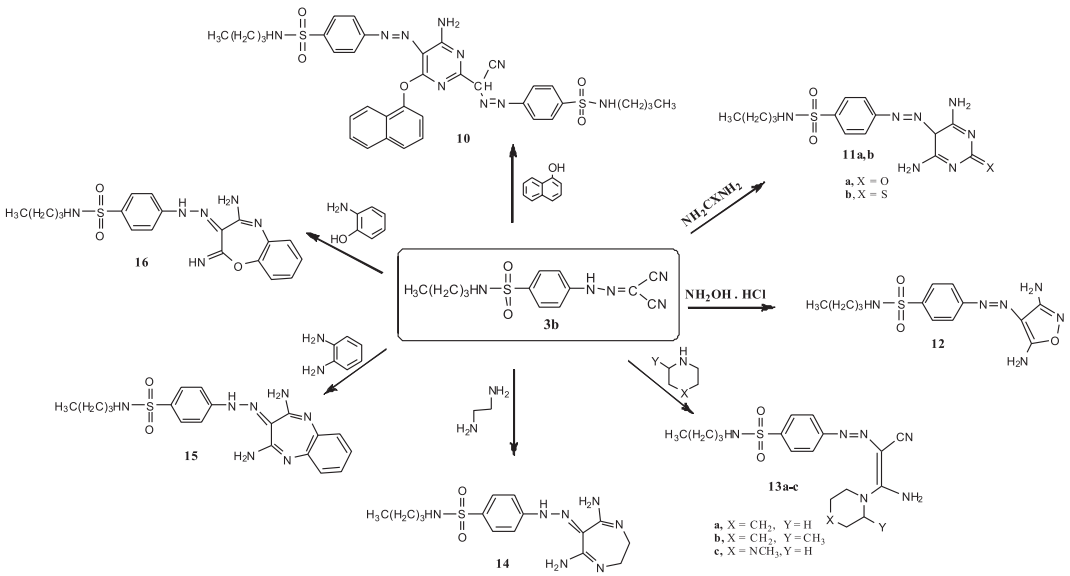
Scheme 2. Synthesis of target compounds **4-9**

4th h. Surprisingly, the replacement of the butyl side chain attached to the benzoyl-pyrazole ring of **8a** compound by a cyclohexyl moiety as in **8b** analogue greatly increased the activity on 1st and 4th h after its administration (% edema inhibition after the 1st h: -60.32, after 4th h: -57.21). At the same time, the attachment the parent azo-benzenesulfonamide nucleus to substituted pyrimidine, diazepine, and pyridopyridazine rings **10, 11a, 14, 18** produced moderate anti-inflammatory activity.

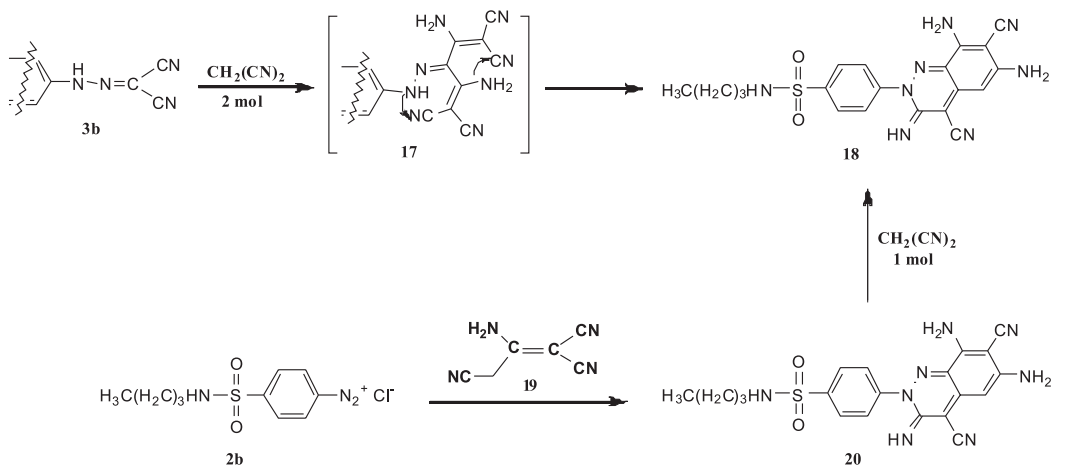
It is noteworthy that the incorporation of the azo-benzenesulfonamide moiety to bulky substituents reduced the anti-inflammatory activity and the duration of action of the tested derivatives. This might be explained by incomplete fitting of the molecules into the active sites of the target enzyme.

Analgesic activity

The pharmacological data of the analgesic activity (Table 3) revealed that the derivatives did



Sheme 3. Synthesis of target compounds **10**-**16**



Scheme 4. Synthesis of target compounds **18**, **20**

Table 1. Anti-inflammatory effects of the tested compounds and celecoxib on percent change of edema in carrageenan-induced edema.

Compound	% Change from baseline			
	1 h	2 h	3 h	4 h
Control	54.8 5± 3.23	67.13 ±4.81	79.48 ± 4.50	83.04 ± 5.12
Celecoxib 50 mg/kg	48.50 ± 6.99	30.82 ±4.19*	24.87 ± 5.00*	19.65 ± 1.33*
1b	50.56 ± 3.34	41.55 ± 4.50	31.43 ± 4.86*	28.12 ± 2.37*
3b	22.41 ± 2.19*	19.36 ± 2.77*	31.19 ± 3.13*	19.00 ± 1.37*
4a	24.25 ± 4.16*	24.63 ± 3.83*	21.08 ± 1.99*	17.01 ± 1.23*
5	47.57 ± 5.56	28.35 ± 4.79*	28.31 ± 2.64*	27.03 ± 1.37*
6a	55.48 ± 9.15	31.35 ± 2.66*	29.20 ± 3.79*	31.52 ± 2.36*
7	71.21 ± 3.13	70.63 ± 6.58	79.42 ± 7.87	59.77 ± 5.26
8a	69.27 ± 5.79	58.92 ± 6.93	60.83 ± 11.32	46.31 ± 4.21
8b	21.76 ± 2.87*	24.86 ± 4.76*	29.77 ± 3.12*	35.53 ± 6.12*
9b	73.90 ± 10.13	76.11 ± 4.50	70.64 ± 11.41	51.78 ± 3.02
10	68.22 ± 5.19	52.27 ± 6.76	60.54 ± 7.01	42.91 ± 3.24*
11a	49.22 ± 5.42	64.52 ± 8.87	57.71 ± 8.50	47.53 ± 4.54
14	55.89 ± 5.65	45.44 ± 2.72	41.20 ± 5.81	36.64 ± 5.39*
16	20.50 ± 2.28*	29.45 ± 6.65*	33.85 ± 11.71*	27.14 ± 2.99*
18	51.20 ± 8.88	63.62 ± 5.96	53.09 ± 11.68	50.86 ± 3.70

Data represent the mean ± standard error of the mean (n = 6). Values represent the mean ± S.E. of six animals for each group. * $p < 0.05$: statistically significant from control. (One way ANOVA followed by Tukey test).

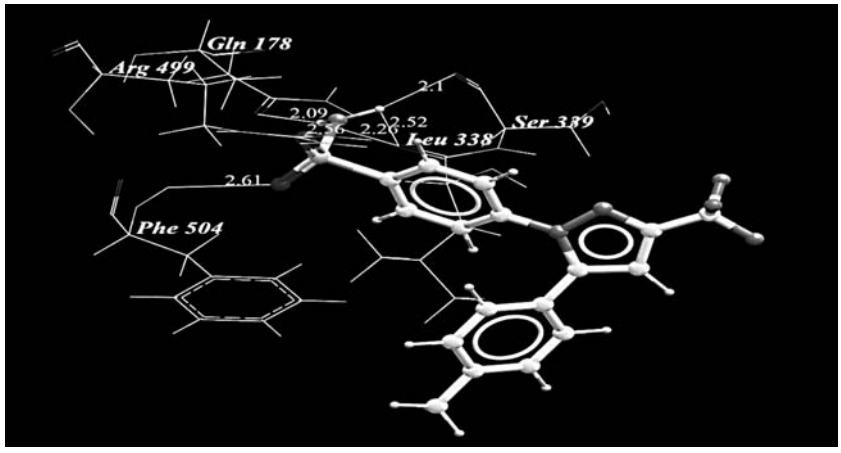


Figure 1. Binding mode of celecoxib with COX-2 showing intermolecular hydrogen bonds

not produce rapid onset of action and the latency times were less than that of the reference drug ranging from 2.74-to 5.18 at 0 min but fortunately, all the derivatives produced long duration of action and their analgesic activities increased with time till 90 min after their administration. The highest analgesic potency, higher than that of celecoxib, was obtained by the benzo[1,4]oxazine derivative **16** (reaction time; 12.04 s at 90 min post compound administration) followed by the cyanoacetyl-pyrazole derivative **7**, benzoylpyrazole derivative **8b** and the pyrimidine derivative **10** (reaction times: 10.06, 10.56 and 10.94 s at 90 min post compounds administration). The rest of the examined compounds produced equipotent analgesic activity to that of celecoxib at 90 min after their administration.

Noticeably, the biological evaluation exhibited that the fused ring benzo[1,4]oxazine in conjugation with azo-benzenesulfoamide nucleus produced dual anti-inflammatory and analgesic potency as in com-

pound **16**. Also, not all the derivatives that produced the highest anti-inflammatory activity exhibited the highest analgesic potency and this can be explained that the analgesic activity of the tested derivatives can be obtained by other mechanisms of actions different from the mechanism of anti-inflammatory effect.

Ulcerogenic activity

The ulcerogenic potential of the tested compounds was evaluated in rats (39). Interestingly, all the tested derivatives showed superior GIT safety profile and exhibited no ulcerogenic effects in comparison to celecoxib which exhibited ulcer severity 19.4 ± 1.652 under the same experimental conditions. These data represented the potential medicinal value of these compounds as anti-inflammatory and analgesic agents, since they have better safety margin than celecoxib on gastric mucosa.

Docking study

To understand both the anti-inflammatory and analgesic data on a structural basis, molecular docking studies were carried out using Mol Soft ICM

Table 2. Percentage of edema inhibition of the tested compounds and celecoxib on carrageenan-induced edema.

Compound%	Inhibition			
	1 h	2 h	3 h	4 h
Celecoxib 50 mg/kg	-11.57	-54.08	-68.70	-76.33
1b	-7.82	-38.10	-60.45	-66.13
3b	-59.14	-71.16	-60.77	-77.12
4a	-55.78	-63.30	-73.47	-79.51
5	-13.27	-57.76	-64.38	-67.44
6a	1.14	-53.29	-63.26	-62.04
7	29.82	5.21	-0.07	-28.02
8a	26.28	-12.23	-23.46	-44.23
8b	-60.32	-62.96	-62.54	-57.21
9b	34.73	13.37	-11.12	-37.64
10	24.37	-22.13	-23.82	-48.32
11a	-10.26	-3.88	-27.39	-42.76
14	1.89	-32.31	-48.16	-55.87
16	-62.62	-56.12	-57.41	-67.31
18	-6.65	-5.22	-33.22	-38.75

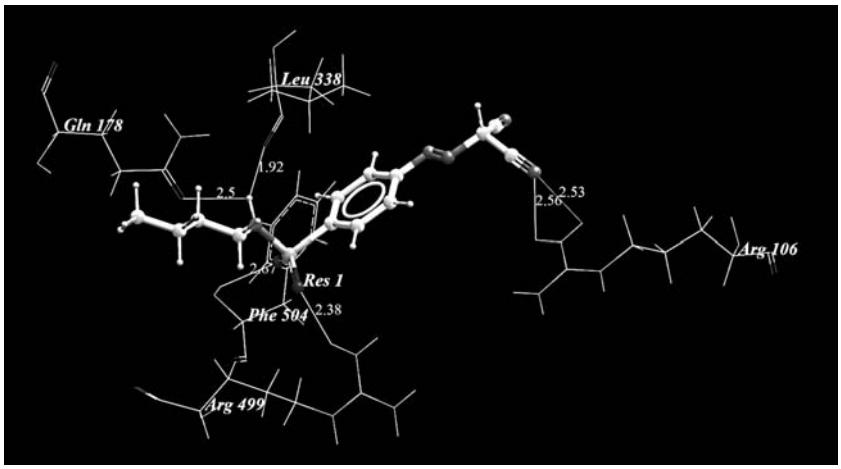


Figure 2. Binding mode of compound **3b** with COX-2 showing intermolecular hydrogen bonds

Table 3. Analgesic activity of the tested compounds and celecoxib by tailflick method.

Compound	Reaction time / min			
	0 min	30 min	60 min	90 min
Control	3.64 ± 0.44	3.76 ± 0.28	4.32 ± 0.51	5.34 ± 0.85
Celecoxib 50 mg/kg	6.14 ± 0.41	6.98 ± 0.53*	6.56 ± 0.54	6.16 ± 0.63
1b	3.86 ± 0.40	4.92 ± 0.45	6.56 ± 0.77	8.42 ± 0.43
3b	3.82 ± 0.31	5.18 ± 0.40	5.58 ± 0.51	6.32 ± 0.77
4a	2.74 ± 0.61	4.24 ± 0.54	5.32 ± 0.76	6.26 ± 0.93
5	3.98 ± 0.30	5.40 ± 0.55	7.08 ± 0.24	7.84 ± 0.46
6a	3.64 ± 0.36	6.38 ± 0.44	5.28 ± 0.37	8.30 ± 0.50
7	4.16 ± 0.75	6.66 ± 0.82	10.64 ± 0.54*	10.06 ± 0.93*
8a	5.18 ± 0.28	7.34 ± 0.46*	7.04 ± 0.53	7.70 ± 0.64
8b	3.92 ± 0.51	6.32 ± 0.64	9.92 ± 0.76*	10.56 ± 0.74*
9b	3.60 ± 0.54	5.50 ± 0.80	7.12 ± 0.66	8.08 ± 0.70
10	4.82 ± 0.81	6.66 ± 0.90	9.60 ± 0.94*	10.94 ± 1.14*
11a	4.68 ± 0.61	6.26 ± 0.31	7.62 ± 0.5	7.92 ± 0.55
14	3.42 ± 0.42	4.24 ± 0.38	6.84 ± 0.68	7.98 ± 0.55
16	3.28 ± 0.37	8.32 ± 1.06*	10.16 ± 1.08*	12.04 ± 1.54*
18	3.40 ± 0.47	4.80 ± 0.82	5.60 ± 0.78	6.76 ± 0.69

Data represent the mean ± standard error of the mean (n = 6). Values represent the mean ± S.E. of six animals for each group.

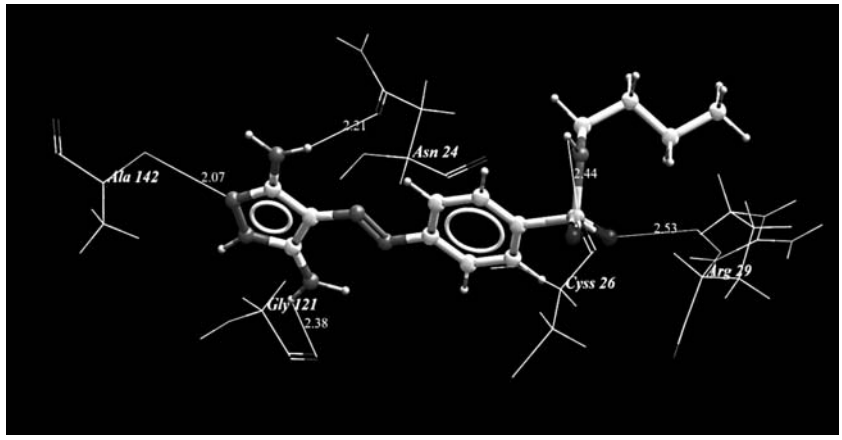


Figure 3. Binding mode of compound **4a** with COX-2 showing intermolecular hydrogen bonds

3.5-0a software. ICM docking is probably the most accurate predictive tool of binding geometry today (40, 41). The aim of the flexible docking calculations is the prediction of correct binding geometry for each binder. The scoring functions and hydrogen bonds formed with the surrounding amino acids of the receptor COX-2 are used to predict the tested compounds binding modes. Celecoxib was used as a reference drug for the anti-inflammatory activity of the new derivatives.

We evaluated the active anti-inflammatory compounds **1b**, **3b**, **4a**, **5**, **6a**, **7**, **8a**, **8b**, **9b, 10**, **11a**, **14**, **16** and **18** through molecular modeling and docking techniques against COX-2 crystal structure, which was downloaded from PDB website PDB id (3LN1). Docking results revealed high affinity of all tested compounds towards COX-2 crystal structure. Binding energy of the docked compounds ranged from -85.81 to -157.72 kcal/mol (Table 4); moreover, all tested compounds

Table 4. The docking energy scores of celecoxib and tested compounds with the amino acid residues of the target enzyme COX-2 forming hydrogen bonds.

Compound	Docking score (kcal/mol)	No. of hydrogen bonds	Amino acid residues forming hydrogen bonds in Å
Ligand (Celecoxib)	-102.24	6	R499 hh11 -- m M o2 : 2.56 F504 hn -- m M o1 : 2.61 Q178 oe1 -- m M h6 : 2.09 L338 o -- m M h5 : 2.52 L338 o -- m M h6 : 2.26 S339 o -- m M h5 : 2.10
3b	-107.81	6	R106 hh11 -- m M n5 : 2.53 R106 hh12 -- m M n5 : 2.56 R499 hh11 -- m M o1 : 2.38 F504 hn -- m M o2 : 2.67 Q178 oe1 -- m M h5 : 2.50 L338 o -- m M h5 : 1.92
4a	-100.27	5	R29 hn -- m M o1 : 2.53 A142 hn -- m M n3 : 2.07 N24 od1 -- m M h7 : 2.21 C26 o -- m M h10 : 2.44 G121 o -- m M h9 : 2.38
5	-102.25	7	N368 hd22 -- m M o1 : 2.68 N368 hd22 -- m M o2 : 2.04 H372 he2 -- m M o1 : 1.61 H372 he2 -- m M o2 : 2.53 Y371 o -- m M h6 : 2.55 H372 nd1 -- m M h12 : 1.85 H374 ne2 -- m M h7 : 2.73
6a	-99.14	5	H75 he2 -- m M n1 : 2.49 Q178 oe1 -- m M h5 : 1.26 Q178 oe1 -- m M h6 : 1.04 S339 o -- m M h7 : 1.40 V509 o -- m M h14 : 2.52
7	-93.15	5	H75 he2 -- m M n2 : 2.38 Q178 oe1 -- m M h10 : 1.18 Q178 oe1 -- m M h11 : 0.95 S339 o -- m M h8 : 1.97 V509 o -- m M h5 : 2.54
8a	-91.28	6	R29 hh11 -- m M o1 : 2.01 Q447 he22 -- m M n3 : 2.43 R455 he -- m M o1 : 2.44 C26 o -- m M h7 : 1.82 R29 o -- m M h13 : 1.95 Q447 oe1 -- m M h5 : 1.87
8b	-108.27	7	Y134 hh -- m M o1 : 1.74 T198 hn -- m M o1 : 2.57 T198 hg1 -- m M o1 : 1.20 T198 hg1 -- m M o2 : 1.71 P204 o -- m M h5 : 2.20 Y371 o -- m M h9 : 2.43 H374 ne2 -- m M h6 : 2.20
9b	-112.86	5	Q27 o -- m M h3 : 2.76 G30 o -- m M h4 : 2.52 G121 o -- m M h15 : 2.73 E451 oe1 -- m M h2 : 2.37 E451 o -- m M h2 : 2.61

Table 4. Cont.

Compound	Docking score (kcal/mol)	No. of hydrogen bonds	Amino acid residues forming hydrogen bonds in Å
10	-157.72	9	C26 hn -- m M o2 : 2.18 C26 hn -- m M o3 : 1.60 Q27 he21 -- m M o2 : 1.73 Q27 he22 -- m M o2 : 2.37 R29 hn -- m M n7 : 2.45 N24 od1 -- m M h5 : 2.53 N24 od1 -- m M h6 : 2.28 N24 o -- m M h29 : 1.53 Q447 oe1 -- m M h5 : 2.20
11a	-106.30	11	R29 hn -- m M o1 : 1.84 R29 hn -- m M n4 : 2.45 C32 hn -- m M o2 : 2.68 Y116 hh -- m M o3 : 2.40 N24 o -- m M h7 : 2.49 C26 o -- m M h6 : 0.90 C26 o -- m M h7 : 1.06 G121 o -- m M h10 : 2.12 E451 oe1 -- m M h8 : 2.39 E451 oe1 -- m M h9 : 2.72 E451 o -- m M h9 : 2.73
14	-100.28	6	R29 hn -- m M o1 : 2.78 A142 hn -- m M n4 : 1.92 C21 o -- m M h5 : 2.60 C21 o -- m M h12 : 1.22 C21 o -- m M h13 : 1.19 N24 od1 -- m M h11 : 2.41
16	-111.58	6	T192 hg1 -- m M o1 : 2.42 T198 hg1 -- m M n3 : 1.53 N368 hd22 -- m M n3 : 2.34 W373 hn -- m M o2 : 1.53 T198 og1 -- m M h6 : 2.23 N368 od1 -- m M h5 : 2.58
18	-110.33	5	R29 hh11 -- m M n7 : 2.12 N24 o -- m M h8 : 2.51 C26 o -- m M h8 : 2.32 Y116 oh -- m M h6 : 2.53 G121 o -- m M h5 : 2.78

showed hydrogen bonding with amino acid residues of COX-2.

Compound **10** showed the highest binding energy with the binding site of COX-2 (-157.72 kcal/mol), while compound **8a** showed the lowest binding energy (-91.28 kcal/mol). Figure 1 shows the binding mode of the original ligand celecoxib into its binding site, while Figures 2 and 3 show the binding modes of compounds **3b** and **4a**, respectively. Docking results of compounds **4a**, which shows the highest anti-inflammatory activity (79.51%) revealed that the compound was nicely fitted into the active site recording high score of binding energy (δG = -100.27 kcal/ mol) forming 5 hydrogen bonds with the amino acids residues at the binding site. The H7 of the amino group N5 of the diamino pyrazole ring forms a hydrogen bond with Asn 24 of COX-2, while the H9 of the second amino group N6 of the diaminopyrazole ring froms a hydrogen bond with Gly 121 of COX-2. Furthermore, the N3 atom of the pyrazole ring forms a hydrogen bond with Ala 142 of COX-2. On the other hand, two hydrogen bonds are formed between H10 of the NH (N7) with Cys 26 and O1 of the sulfonamide group with Arg 29 of COX-2 while the phenyl ring settles in a hydrophobic cavity. From the above mentioned data, it is obvious that compound **4a** binds to the active site of COX-2 in a dif-

ferent mode compared to the reference drug celecoxib. More interestingly, for compound **3b** which shows the second highest anti-inflammatory activity (-77.11%), docking result (Fig. 2) revealed a mode of binding to the active site of COX-2 very similar to the reference drug celecoxib. They both formed hydrogen bonding between the sulfonamide group and Gln 178, Leu 338, Arg 499, Phe 504 amino acids in the binding site of COX-2. In addition, compound **3b** formed two extra hydrogen bonds between the terminal CN group and Arg 106 of COX-2 binding site. This result suggests that the compound **3b** binds to the active site of COX-2 in a similar mode to that of reference drug celecoxib.

CONCLUSIONS

This work represented the synthesis of different derivatives comprising benzene sulfamyl nucleus in conjugation with different heterocyclic functionalities such as: pyrazole, pyrimidine, isoxazole, diazepine, benzodiazepine, benzoxazepine and pyrido-pyridazine. Some of the newly synthesized compounds (14 compounds) were selected for their evaluation as anti-inflammatory and analgesic agents.

The highest anti-inflammatory potency that is equipotent to that of celecoxib was gained by derivatives **1b**, **3b**, **4a**, **5** and **16**. The rest of the derivatives appeared to be moderately active as anti-inflammatory agents. The analgesic evaluation of the tested compounds showed that derivatives **16**, **7**, **8b**, **10** exhibited much higher analgesia in the experimental rats than celecoxib after 90 min post their administration, while the rest of the tested derivatives produced equipotent activity to that obtained by the reference drug. It has been noticed that derivative **16** is of dual anti-inflammatory and analgesic activity. It is noteworthy that all of the examined compounds exhibited superior GIT safety profile in the experimental rats better than celecoxib.

Molecular docking studies showed that compounds **3b, 4a, 5, 8a, 8b, 10, 11a, 14, 16, 18** represented good fitting into the active sites of COX-2 enzyme, forming different hydrogen bonds with the amino acid residues at the active sites of the enzyme. There is a good relation between the resultant anti-inflammatory biological data and the molecular docking results since it has been observed that compound **4a** of the highest anti-inflammatory potency showed the best fitting and highest score of binding energy (-100.27 kcal/mol), forming 5 hydrogen bonds with amino acid residues at COX-2 binding sites.

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SYNTHETIC DERIVATIVES OF ISOQUINOLINE, DICARBOXYLIC ACID IMIDES AND THIOIMIDES AS BIOACTIVE COMPOUNDS

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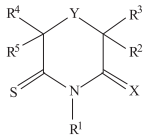
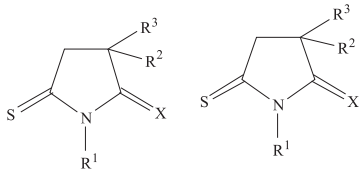
Abstract: This study is a continuation of a research program aimed at identifying potent drugs against bacterial infections, in which a series of organic compounds: dicarboxylic acid imides and thioimides, isoquinoline derivatives and open chain compounds, were examined for antimicrobial properties against *Staphylococcus aureus* and *Escherichia coli*. In effect of this investigation, the most active compounds (**35**-**40**, **47**) were selected for *in vitro* tests against fourteen clinically important pathogenic isolates, the methicillin resistant *Staphylococcus aureus* (MRSA) and several reference Gram-negative bacteria: *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Stenotrophomonas maltophilia*, and *Acinetobacter baumannii*. The obtained data revealed that seven compounds (three dithioimides, **35**, **39**, **47,** and four thioimides, **36**-**38**, **40**) exhibit effective antibacterial activity against the tested *Staphylococcus aureus* MSSA and MRSA strains. Among them, dicarboxylic acid thioimides **37** and **38** were proven to be the most active.

Keywords: antibacterial activity, DD-peptidase, dicarboxylic acid imides, isoquinoline, thioimides

The discovery and medicinal use of antibiotics in the 1950s have undoubtedly conferred one of the greatest benefits on mankind. Over the following 10–15 years, the average lifetime of the human population increased significantly, some infectious diseases almost disappeared, while several neoplastic and viral diseases became controllable (1). The use of antibiotics, often excessive and indiscriminative, both in medicine and veterinary science has contributed to the emergence of drug resistant organisms. In effect, antimicrobial drug resistance has become a growing problem worldwide. Infections caused by resistant pathogens trigger increased mortality and morbidity among human and animal populations. Moreover, pathogenic microorganisms, including *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Clostridium difficile*, contribute to many hospital-acquired infections. Therefore, iden-



Compound **35** (R^1 = H, R^2 = CH_3, R^3 = CH_3, R^4 = CH_3, R^5 = CH_3, X = S, Y = C),
Compound **36** (R^1 = H, R^2 = CH_3, R^3 = CH_3, R^4 = CH_3, R^5 = CH_3, X = O, Y = C),
Compound **53** (R^1 = C_6H_4-CH_3, R^2 = H, R^3 = H, R^4 = H, R^5 = H, X = O, Y = S),
Compound **54** (R^1 = C_6H_4-Cl, R^2 = H, R^3 = H, R^4 = H, R^5 = H, X = O, Y = S).



Compound **37** (R^1 = H, R^2 = CH_3, R^3 = CH_3, X = O),
Compound **38** (R^1 = H, R^2 = CH_3, R^3 = H, X = O),
Compound **39** (R^1 = C_6H_5, R^2 = H, R^3 = H, X = S),
Compound **40** (R^1 = C_6H_5, R^2 = H, R^3 = H, X = O),
Compound **47** (R^1 = H, R^2 = CH_3, R^3 = CH_3, X = S).

Figure 1. Chemical structures of active compounds (**35**–**40**, **47**, **53**, **54**)

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Table 1. Antibacterial activity (MIC) and systematic names of compounds **1**-**54**.

Compound		MIC (μg/mL)			Ref.
No.	Name	*S. aureus* ATCC 29213 (MSSA)	*S. aureus* ATCC 43300 (MRSA)	*E. coli* ATCC 25922	
1	6,7-Dimethoxy-1-phenyl-1,2,3,4-tetrahydroisoquinoline	>256	-	>256	13
2	6,7-Dimethoxy-2-methyl-1-phenyl-1,2,3,4-tetrahydroisoquinoline	>256	-	>256	14
3	1-[1-(3,4-Dimethoxy-phenyl)-6,7-dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl]-ethanone	>256	-	>256	15
4	Cyclohexanecarboxylic acid (2-cyclohex anecarbonyl-1,2,3,4-tetrahydroisoquinolin-1-ylmethyl)-amide	>256	-	>256	16
5	(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-methanol	>256	-	>256	17
6	1-Heptadecyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline	>256	-	>256	18
7	N-[3-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-propyl]-guanidine	>256	-	>256	19
8	2-[3-(6,7-Dimethoxy-1,2,3,4-tetrahydroisoquinolin-1-yl)-propyl]-isoindole-1,3-dione	>256	-	>256	19
9	6,7-Dimethoxy-1-phenyl-3,4-dihydroisoquinoline	>256	-	>256	14
10	(6,7-Dimethoxy-3,4-dihydroisoquinolin-1-yl)-(3,4-dimethoxy-phenyl)-methanone	>256	-	>256	20
11	2-(3,4-Dihydroisoquinolin-1-ylmethyl)-isoindole-1,3-dione	>256	-	>256	16
12	2,3,10,11-Tetramethoxy-5,8,13,13a-tetrahydro-6H-isoquino[3,2-a]isoquinoline	>256	-	>256	21
13	2,3,10,11-Tetramethoxy-8-methyl-5,6-dihydroisoquino[3,2-a]isoquinolinylium acetyl sulfate	>256	-	>256	22
14	2,3-Dimethoxy-5,6-dihydroisoquino[3,2-a] isoquinolin-8-one	>256	-	>256	23
15	2,3,10,11-Tetramethoxy-5,6,8,13-tetrahydroisoquino[3,2-a]isoquinolinylium chloride	>256	-	>256	24
16	2,3,10,11-Tetramethoxy-5,6-dihydroisoquino [3,2-a]isoquinolinylium chloride	>256	-	>256	25
17	8,9-Dimethoxy-1,2,3,5,6,10b-hexahydropyrrolo [2,1-a]isoquinoline	>256	-	>256	26
18	9,10-Dimethoxy-1,3,4,6,7,11b-hexahydro-2H-pyrido[2,1-a]isoquinoline	>256	-	>256	26
19	8,9-Dimethoxy-2,3,5,6-tetrahydro-1H-pyrrolo[2,1-a]isoquinolinylium chloride	>256	-	>256	26

Table 1. Cont.

Compound		MIC (μg/mL)			Ref.
No.	Name	*S. aureus* ATCC 29213 (MSSA)	*S. aureus* ATCC 43300 (MRSA)	*E. coli* ATCC 25922	
20	9,10-Dimethoxy-1,2,3,4,6,7-hexahydropyrido[2,1-a]isoquinolinylium chloride	>256	-	>256	26
21	4-Methyl-6-phenyl-1,2,3,4-tetrahydroquin olizinylium bromide	>256	-	>256	27
22	4-(4-Methoxyphenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
23	4-(2,4-Dichlorophenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
24	4-Benzo[1,3]dioxol-5-yl-thiomorpholine-3,5-dione	>256	-	>256	28
25	4-(2,5-Diethoxyphenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
26	4-(4-Nitrophenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
27	4-(4-Chlorophenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
28	4-(2,5-Dimethylphenyl)-thiomorpholine-3,5-dione	>256	-	>256	28
29	4-p-Tolyl-thiomorpholine-3,5-dione	>256	-	>256	28
30	3,3-Dimethylpiperidine-2,6-dione	>256	-	>256	29
31	6-Hydroxy-3,3,4,4-tetramethyl-6-phenyl-piperidin-2-one	>256	-	>256	29
32	1-Benzyl-3,3-dimethylpiperidine-2,6-dione	>256	-	>256	30
33	1-Benzyl-3,3,4,4-tetramethylpiperidine-2,6-dione	>256	-	>256	31
34	3,3,4,4-Tetramethyl-6-phenyl-3,4-dihydro-1H-pyridin-2-one	>256	-	>256	29
35	3,3,5,5-Tetramethylpiperidine-2,6-dithione	32	32	>256	32
36	3,3,5,5-Tetramethyl-6-thioxopiperidin-2-one	32	64	>256	32
37	3,3-Dimethyl-5-thioxopyrrolidin-2-one	8	32	>256	33
38	3-Methyl-5-thioxopyrrolidin-2-one	8	8	>256	32
39	1-Phenylpyrrolidine-2,5-dithione	16	16	>256	34
40	1-Phenyl-5-thioxopyrrolidin-2-one	16	16	>256	35
41	1-(2-Chloro-benzyl)-3,3-dimethylpyrrolidine-2,5-dione	>256	>256	>256	31
42	1-(4-Chlorobenzyl)-3,3-dimethylpyrrolidine-2,5-dione	>256	>256	>256	31
43	3,3,4,4-Tetramethylpyrrolidine-2,5-dione	>256	>256	>256	36
44	3,3,4,4-Tetramethylpyrrolidine-2,5-dithione	>256	>256	>256	37
45	3-Phenyl-5-thioxopyrrolidin-2-one	>256	>256	>256	
46	3,3,4,4-Tetramethyl-5-thioxopyrrolidin-2-one	>256	>256	>256	37
47	3,3-Dimethylpyrrolidine-2,5-dithione	32	64	>256	38
48	2,2,3,3-Tetramethylpentanedioic acid	>256	-	>256	29
49	2,2,4,4-Tetramethylpentanedioic acid	>256	-	>256	39
50	4-Benzylcarbamoyl-2,2-dimethylbutyric acid	>256	-	>256	31
51	4-Oxo-4-phenyl-N-(1-phenylethyl)-butyramide	>256	-	>256	40
52	N-Benzyl-4-oxo-4-phenyl-butyramide	>256	-	>256	41
53	5-Thioxo-4-p-tolylthiomorpholin-3-one	128	128	>256	42
54	4-(4-Chlorophenyl)-5-thioxo-thiomorpholin-3-one	256	256	>256	42

[1)] MIC of the reference compound: penicillin G against S. aureus ATCC 43300 - 5.0 (μg/mL).

tification and development of new antibiotics, especially those with new modes of action, is imperative to help treat these infections (2, 3).

In this work, we present a study on antibacterial properties of a series of 54 compounds, comprising dicarboxylic acid imides and thioimides, many isoquinoline analogs, as well as open chain compounds, obtained in our lab by chemical synthesis (Table 1, Fig. 1).

Structural similarities of our synthesized compounds to already known penicillin-binding proteins/DD-carboxypeptidases/DD-transpeptidase inhibitors with γ-, or δ-lactam structure, such as lactavicin (4, 5), encouraged us to perform enzymatic assays using DD-peptidase 64-575 (6). By this approach, we characterized the inhibitory activity of some compounds on DD-carboxypeptidase.

EXPERIMENTAL

Chemistry, general experimental procedures

The NMR spectra were recorded on a Bruker AVANCE spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. The spectra were measured in $CDCl_3$ and are given as δ values (in ppm) relative to TMS. Mass spectra were collected on Quattro LC Micromass and LCT Micromass TOF HiRes apparatus with ESI(+) ionization. Melting points were determined with EZ-Melt melting point apparatus and are uncorrected. TLC analyses were performed on silica gel plates (Merck silica gel 60 F254) and visualized using UV light. Column chromatography was carried out at atmospheric pressure using silica gel 60 (230–400 mesh, Merck). Solvents used in the reactions were anhydrous. Reactants were purchased from Sigma-Aldrich.

All compounds presented in this manuscript were obtained in our group according to our own or literature methods. The references are listed in Table 1.

Compound **45** was obtained by the following procedure (see Fig. 2). Acid anhydride (1.0 mmol) was mixed with aqNH_3 (10 mL) for 1 h. The solvent was evaporated and the residue was heated at 180°C for 2 h. The crude imide was purified by column chromatography on silica gel using mixture hexane/EtOAc as eluent.

The imide (1.0 mmol) and the Lawesson's reagent (0.5 mmol) were dissolved in dry THF (30 mL) and mixed under argon atmosphere for 2 days. The solution was evaporated to dryness. The crude monothioimide was purified by column chromatography on silica gel using mixture hexane/EtOAc as eluent.

Spectral data of **45**: ^{1}H NMR (300 MHz, $CDCl_3$, δ, ppm): 9.56 (brs, 1H), 7.22-7.38 (m, 5H), 4.10 (dd, J = 3 Hz, 1H), 3.63 (dd, J = 9 Hz, 1H), 3.25 (dd, J = 6 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$, δ, ppm): 210.12, 179.75, 136.55, 129.48, 128.35, 127.63, 48.81. HRMS: m/z $[M + Na]^+$ calcd. for $C_{10}H_9NOSNa$: 214.0303; found: 214.0312.

Evaluation of antibacterial activity

Antibacterial activity of compounds was tested by liquid microdilution method (7). The antimicrobial spectrum of 21 derivatives of isoquinoline, 16 dicarboxylic acid imides and 12 thioimides, and 5 open chain compounds were evaluated by the minimal inhibitory concentrations (MIC) using the serial two-fold dilution method under standard conditions as described in the Clinical and Laboratory Standards Institute (CLSI) reference method M07-A8. A panel of Gram-positive bacterial strains was used: *Staphylococcus aureus* ATCC® 29213 (sensitive to methicillin, (MSSA)) and *Staphylococcus aureus* ATCC® 43300 (resistant to methicillin, (MRSA)). Gram-negative, *Escherichia coli* ATCC® 25922™ was also tested. Additionally, the active compounds were tested on strains*: Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* subsp. *pneumoniae* ATCC 13882, *Stenotrophomonas maltophilia* ATCC 13637, *Acinetobacter baumannii* ATCC 19606. Fourteen clinical isolates of methicillin resistant *Staphylococcus aureus* (MRSA) obtained from Department of Pharmaceutical Microbiology of Medical University of Warsaw, Poland were also used. The clinical isolates were collected from various patients hospitalized in several clinics.

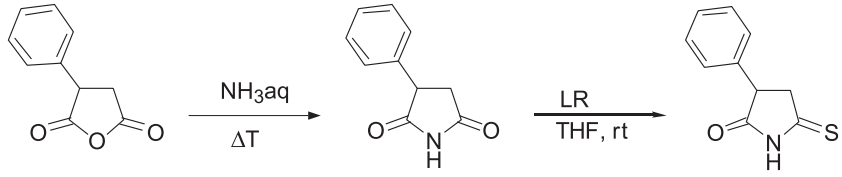

Figure 2. Scheme of synthesis of compound **45**

The bacteria *Staphylococcus aureus, Escherichia coli*, *Pseudomonas aeruginosa* were cultivated on tryptic soy agar (TSA; Oxoid). *Klebsiella pneumonia, Acinetobacter baumannii, Proteus vulgaris and Stenotrophomonas maltophilia* were cultivated onto nutrient agar (Oxoid). All strains were incubated for 24 h at 37°C.

Reference method (broth microdilution susceptibility test) was as follows: All compounds were dissolved in DMSO. A series of the twofold compounds dilutions were diluted with cation-adjusted Mueller–Hinton broth (CAMHB). Aliquots (95 µL) were dispensed into microdilution sterile plates (Mar-Four). Then, 5 µL of bacteria inoculum, containing 5×10^4 CFU/mL, was added. The final concentration of compounds ranged from 256 to 4 µg/mL all in two-fold dilution steps. The experiments for each sample were conducted in triplicate. Penicillin G was used as control (from 10–0.16 µg/mL). The plates were incubated at 37°C (35°C) for 18 to 24 h depending on bacterial strain. Results were obtained with the use of Spectrostar Omega (BMG Labtech), absorbance was measured at λ = 540 nm and 595 nm. MIC was defined as the lowest drug concentration that reduced growth by 100%.

Assay of 35-40 and 47 compounds inhibitory activity on DD-carboxypeptidase 64-575 (DD-peptidase 64-575) (6, 8, 9)

Under the procedures described previously (10), the inhibitory effect of DD-carboxypeptidases activity was measured. Thioester C_6H_5-CO-NH-(D)CH(CH_3)-CO-S-CH_2-COOH was used as a substrate. Dicarboxylic acid dithioimides (**35**, **39, 47**) and structurally related dicarboxylic acid thioimides (**36**-**38**, **40**) were examined.

The amount of 100 µL of the reaction mixture contained 7 µL of DD-peptidase 64-575, 5 µL of 10 mM substrate solution (thioester: benzoyl-D-alanyl-thioglycollate (synthesized at Siedlce University)), 5-10 µL of fresh inhibitor's solution (in methanol) at various concentration and phosphate buffer, pH 8.0 or destilled H_2O. Hydrolysis of thioester was monitored spectrophotometrically (Jasco V-630, Japan) at 250 nm in 37°C during 3000 s. The IC_{50} values of these compounds for DD-peptidases 64-575 are presented in Table 2.

Assay of β-lactamase activity

Inhibition of penicillinase (Penase, $5 \cdot 10^6$ IU/mL) by **35**-**40** and **47** compounds was evaluated based on the method described in the literature (11). A mixture of β-lactamase and selected compounds in 0.1 M phosphate buffer pH 7.0 was incubated for 30 min at 37°C, then nitrocephin was added, and the mixture was incubated for additional 10 min at 37°C. Subsequently, absorbance was measured at 482 nm.

Hemolysis

Hemolysis test was evaluated following the literature method (12). Human red blood cells obtained from healthy volunteers were prepared in phosphate buffered saline (PBS, pH 7.4). Prepared suspension of 1% hematocrit was incubated with serial concentrations (from 0.01 to 1.2 mM) of selected, compounds (**35**-**40**, **47**) for 30 min at 23°C. After centrifugation (4,000 × g, 5 min), the absorbance of supernatant was measured at 540 nm (Jasco V-630). A value of 100% hemolysis was determined by incubation of erythrocytes with double-distilled water (30 min at 23°C). In a control experiment, cells were incubated in buffer without tested compounds and absorbance at 540 nm value was used as a blank.

RESULTS AND DISCUSSION

Preparation of chemical compounds for biological tests

Organic compounds tested in this study were obtained by chemical synthesis according to our own or literature methods. The appropriate references are listed in Table 1.

Most of these compounds belong to chemical groups comprising known compounds which biological activity has been already described. In general, they can be classified to three main chemical groups: isoquinoline derivatives (compounds **1-20**), dicarboxylic acid imides (compounds **22**-**33**, **41**-**43**) and thioimides (compounds **35**-**40**, **44**-**47**, **53**, **54**), (Table 1).

Antimicrobial activity

We screened a total of 54 compounds for *in vitro* antibacterial activity against the Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* bacteria using the broth microdilution susceptibility test. Results are reported as the minimum inhibitory concentration (MIC) in µg/mL in comparison to the reference antibacterial agent - penicillin G, and are summarized in Table 1.

Initially, we examined the antimicrobial activities of isoquinoline alkaloids. Known isoquinoline analogs with the structure of natural or synthetic origin, exhibit antibacterial properties against *Escherichia coli, Pseudomonas aeruginosa, Proteus mirabilis, Klebsiella pneumoniae, Acinetobacter bau-*

mannii, Staphylococcus aureus, Bacillus subtilis and antifungal against *Candida albicans* (43-47). However, in our experiments, among 20 investigated synthetic isoquinoline alkaloids (**1**-**20**)**,** including tetrahydroisoquinoline derivatives (**1**-**8**, **17**, **18**), dihydroisoquinoline derivatives (**9**-**11**, **19**, **20**), protoberberine analogs (**12**-**16**) and tetrahydroquinolizidine (**21**), none of them exhibited antimicrobial activity (MIC > 256 μg/mL) in relation to *S. aureus* or *E. coli* (Table 1). Various substituents on the isoquinoline core, e.g., phenyl ring at C1 (**1**, **2, 9**), guanidine at C1 (**7**), isoquinoline (**12**-**16**), pyrrolo group (**17**-**19**) or pyrido group (**18**, **20**), did not affect the lack of antibacterial activity of these compounds.

Based on the literature data, imides and their N-substituted analogs were found active towards bacteria from the ATCC collection (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*) (48). Contrarily, analogous compounds (imides: **30**, **31**, **34**, **43** and N-substituted analogs: **32**, **33**, **41**, **42**) obtained in our lab proved to be inactive under the tested conditions (Table 1).

Thionation of the amide moiety in imides allows designing thioimides that exhibit significantly enhanced antibacterial activity compared to the parent systems (49). Recently, such imides proved to be important pharmacophores in derivatives displaying antimicrobial and antitumor activity (50). Out of the 54 tested compounds, nine (thioimides **36-38**, **40** and dithioimides **35**, **39**, **47)** presented significant antibacterial activities against *Staphylococcus aureus* (MIC 8-64 μg/mL) (Table 1, Fig, 1). Among them, the most active antibacterial agents were five-member ring thioimides **37** and **38,** with MIC of 8 μg/mL and 32 μg/mL respectively, against *S. aureus* MSSA and MRSA. Based on our findings, structure modification of the six member heterocyclic thioimide of piperidine to a five member het-

Table 2. Biological activity of selected compounds: extended spectrum of antibacterial activity (MIC) and inhibition of DD-peptidase 64-575.

Bacterial strain/ MIC (μg/mL)	**35**	**36**	**37**	**38**	**39**	**40**	**47**
P. aeruginosa ATCC 27853	>256	>256	>256	>256	>256	>256	>256
P. vulgaris ATCC 6896	>256	>256	>256	>256	>256	>256	>256
K. pneumoniae ATCC 13882	>256	>256	>256	>256	>256	>256	>256
S. maltophilia ATCC 13637	>256	>256	128	256	>256	256	-
A. baumannii ATCC 19606	>256	>256	128	>256	>256	>256	128
Clinical isolates of *S. aureus* (MRSA) 304	32	64	16	32	64	64	-
305	256	64	32	32	64	64	128
306	>256	256	32	128	256	>256	256
308	128	128	32	32	64	64	128
309	256	64	16	16	128	64	256
329	256	64	32	32	64	64	256
330	32	32	16	16	32	32	32
333	16	32	16	16	32	32	16
334	64	64	32	32	64	64	16
353	32	32	16	16	32	32	32
354	32	32	16	16	32	32	32
355	32	32	8	16	32	32	32
356	>256	128	32	32	64	64	256
359	>256	256	128	128	256	64	256
Inhibition of DD-peptidase 64-575 IC_{50} (mM)	>2.29	1.60	0.55	0.50	0.18	2.30	0.47

erocyclic thioimide of pyrrolidine (**37**-**47**) improved the antibacterial activity of the compounds. Furthermore, compounds **41**-**43** with dioxo substituents at position -2,5 showed lack of activity, while compounds **39**, **47,** possessing dithioxo substituents at position 2,5 with a phenyl ring attached to N or dimethyl groups at position 3, respectively, exhibited increased antibacterial activity (MIC 16, 32 μg/mL). Moreover, compounds **37**, **38**, **40** with oxo and thioxo substituents at position 2,5 and dimethyl substituent at position 3, or methyl substituent at this position, or phenyl ring at position 1, respectively, displayed almost the same antibacterial activity. Additionally, compounds **35**-**40** and **47**, found active on *S. aureus*, were further tested on the following strains: *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Stenotrophomonas maltophilia* and *Acinetobacter baumannii*. These compounds were not active (MIC > 256 μg/mL) on Gram-negative bacteria (Table 2).

Further on, compounds **35**-**40** and **47** were selected for antibacterial activity tests *in vitro* against clinically important pathogenic isolates of methicillin resistant *S. aureus* (MRSA). Results of this investigation, summarized in Table 2, show that 79 and 93% of the clinical isolates exhibited sensitivity to dithioimide **35** (MIC 16-256 μg/mL) and thioimide **40** (MIC 32-64 μg/mL), respectively. Moreover, 100% of clinical isolates showed sensitivity to thioimides **36**-**38** and dithioimide **39** (MIC 8-256 μg/mL). As shown in Table 2, thioimides **37** and **38** display potent antibacterial activities against MRSA, suggesting that further development of such compounds may be of interest.

The thiomorpholine-3,5-dione (3-thiaglutaric acid imide) structural motif is present in many pharmacologically active compounds. Synthesis of such derivatives was undertaken to evaluate their potential use as hypnotics, sedatives and anticonvulsants (51). In our study, only two (**53**, **54**) of 10 3-thiaglutaric acid imides (**22**-**29**, **53**, **54**) exhibited antibacterial activity, yet at low level, with MIC of 128 and 256 μg/mL, respectively (Table 1). In further assays, open chain compounds (**48**-**52**) showed no antibacterial activity (MIC > 256 μg/mL), (Table 1).

Inhibitory effect of compounds 35-40, 47 on DD-carboxypeptidase 64-575

Six compounds (**36**-**40**, **47**) were shown to exhibit inhibitory effects on DD-carboxypeptidase 64-575. Concentrations of these compounds necessary to inhibit DD-carboxypeptidase by 50% (IC_{50}) were calculated (Table 2). Obtained IC_{50} values were compared with IC_{50} of β-lactam antibiotics, e.g., IC_{50} of azlocillin, aztreonam and cefsulodin for DD-carboxypeptidase 64-575 were 0.22 mM, 1.6 mM and 0.4 mM, respectively. The IC_{50} value of thioimides **37** and **38** for DD-carboxypeptidase 64-574 was comparable to the IC_{50} value of cefsulodin for this enzyme (9). Until today, there have not been any described inhibitors of DD-peptidases from the group of the dicarboxylic acid thioimides. Supposedly, thioimides **37** and **38** may inhibit cell wall biosynthesis by disturbing cross-linking of peptidoglycan, analogously to the mechanism of action of β-lactam antibiotics.

Inhibitory effect of compounds 35-40 and 47 on β-lactamase

As the examined alkaloids have been shown to inhibit the DD-peptidase 64-575 activity similarly to the clavulanic acid, which is a known inhibitor of β-lactamases, an inhibition test was performed using this enzyme (11). However, in contrast to the clavulanic acid, all of the tested compounds did not reveal inhibitory activity against β-lactamase from *Bacillus cereus*.

Hemolysis

The most active compounds, 3 dithioimides, **35**, **39**, **47,** and 4 thioimides, **36**-**38**, **40,** were examined *in vitro* for hemolytic activity. The conducted assays revealed that the tested compounds did not exhibit any hemolytic activity (up to 1.2 mM) on human erythrocytes.

CONCLUSION

The antimicrobial activity of 54 synthetic compounds representing various groups, such as isoquinoline analogs, dicarboxylic acid imides and thioimides, together with open chain compounds and thioimides of thiaglutaric acid, was investigated. Additionally, DD-peptidase 64-575 and β-lactamase inhibitory activity as well as hemolytic properties of the most active compounds were studied. The obtained data revealed that 7 compounds, 3 dithioimides, **35**, **39**, **47,** and 4 thioimides, **36**-**38**, **40,** exhibited antimicrobial activity against the tested *S. aureus* strains. Among the investigated compounds, derivatives of dicarboxylic acid, thioimides **37** and **38,** bearing, respectively, the dimethyl- or methyl- group at position -C3, were found to be the most potent. It is for the first time that their biological properties have been determined. For better evaluation of the properties of these compounds, further microbiological studies should be conducted.

Acknowledgment

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NMR, HRMS spectra of compound **45** are available from the corresponding author.

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IN SILICO ADME STUDIES OF POLYAMINE CONJUGATES AS POTENTIAL ANTICANCER DRUGS

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Abstract: The ADME properties and drug-likeness of several polyamine derivatives containing dimeric phthalimide, quinoline, cinnoline and chromone moieties with three different polyamines: 1,4-bis(3-aminopropyl)piperazine, 4,9-dioxa-1,12-dodecanediamine and 3,3-diamino-*N*-methyldipropylamine as linkers were presented. These compounds are structurally related to DNA bisintercalators, a group of agents with anticancer activity. Their biological properties were previously assessed *in vitro* in a highly aggressive melanoma cell line A375. Current studies evaluate their potential to be qualified as drugs by performing *in silico* ADME screening. The research is carried out using Discovery Studio 3.0 ADMET protocol obtained from Accelrys and might be useful for further developing and optimization of potential anticancer drugs.

Keywords: ADME, drug-likeness, polyamine conjugates

Improving the pharmacokinetic profile of drug candidates is an extremely important element in the process of introducing them to the treatment. Problems with absorption, distribution, metabolism and elimination (ADME) have been recognized as a major reason for the failure in late-stage of pharmaceutical research and development process (1). Even if a drug lead exhibits excellent activity *in vitro*, poor biopharmaceutical profile will terminate its development (2). As a result, a new trend in modern drug discovery has been focused on parallel evaluation of efficacy and ADME properties of drug candidates at the earliest stages in their development (3). In addition to the advancements in experimental assays, there has been a considerable interest in computational methods for predicting ADME properties (4).

In silico methods have become a useful tool enabling better understanding of the process underlying ADME behavior in humans at very early stages of drug development. Furthermore, computer aided ADME screening helps to identify compounds with acceptable pharmacokinetic properties or indicate potential weaknesses which need to be improved by chemical modifications (5).

In case of anticancer agents, it is said that unfavorable ADME characteristics are not the predominant cause of elimination from further study, because high activity is of greater importance. In the absence of therapeutic alternative, relatively high degree of side effects or not fully optimized pharmacokinetic profile (e.g., lack of oral dosage form) is accepted. On the other hand, drug must reach sufficient levels in the site of action, which is extremely reliable with appropriate pharmacokinetic profile (6). Nowadays, most anticancer drugs are administered intravenously (*i.v.*) because it provides immediate and complete bioavailability. It became of particular importance to optimize oral bioavailability of anticancer drugs in order to enable patients to have more comfortable life and to increase cost-effectiveness of chemotherapy (7).

According to the paradigm that parallel optimization of ADMET properties along with synthesis and evaluating cytotoxic activity *in vitro*, offers a greater chance of identifying a high quality clinical candidates (3, 8) and taking into consideration our earlier studies on potential anticancer drugs (9, 10), we decided to evaluate their potential to be qualified as drugs by performing *in silico* ADME screening. From a wide variety of available ADMET models with different levels of complexities, Discovery Studio 3.0 ADMET protocol obtained from Accelrys was chosen to calculate and to predict the ADMET properties of synthesized polyamine derivatives containing dimeric phthalimide **1**, **2**, quinoline **3-6**, cinnoline **7-10** and chromone **11**, **12** moi-

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eties with three different polyamines: 1,4-bis(3-aminopropyl)piperazine (**a**), 4,9-dioxa-1,12-dodecanediamine (**b**) and 3,3-diamino-*N*-methyldipropylamine (**c**) as linkers. These agents are structurally related to bisintercalators, a group of compounds that interact reversibly with the DNA double helix and share common structural features such as the presence of two planar, polycyclic aromatic systems separated by a spacer chain long enough to enable double intercalation according to the neighbor exclusion principle (11, 12). Their biological activity was investigated *in vitro* on the highly aggressive melanoma cell line A375. The most promising entities were among dimeric quinoline and chromone derivatives which efficiently inhibited cell proliferation and induced cell cycle disturbances. Additionally, the most potent chromone derivative diminished the adhesive potential of melanoma cells (9, 10).

EXPERIMENTAL

Drug-likeness of examined compounds was evaluated using The Filter by Lipinski and Veber Rules protocol (13). The default values for the filters were set according to the published articles (14, 15). ADME studies were performed in Discovery Studio 3.0 (DS®3.0, Accelrys Software Inc.) using ADMET Protocol, which includes models for intestinal absorption (HIA), aqueous solubility, blood brain barrier penetration (BBB), plasma protein binding (PPB), cytochrome P450 2D6 inhibition and hepatotoxicity. Parameters that were calculated using DS®3.0 include: atom based LogP98 (ADMET_AlogP98), polar surface area (ADMET_PSA_2D), the base 10 logarithm of the molar solubility as predicted by the regression (ADMET_Solubility_log S_w), base 10 logarithm of [(brain concentration) / (blood concentration)] as predicted by a robust (least-median-of-squares) regression derived from literature *in vivo* brain penetration data (ADMET_BBB), number of hydrogen bond donors (Num_H_Donors), number of hydrogen bonds acceptors (Num_H_Acceptors), number of rotatable bonds (Num_Rotatable_Bonds), molecular weight (Mw), ADME_EXT_CYP2D6 – Bayesian score from the model, ADME_EXT_HEPATOTOXIC - Bayesian score from the model, ADMET_EXT_PPB - Bayesian score from the model (13).

The polyamine derivatives **1**-**12** introduced into Discovery Studio® 3.0 for carrying out ADME screening are presented in Table 1.

RESULTS

In this research, the polyamine derivatives **1**-**12** with well-defined structures and exhibiting antiproliferative activity against melanoma cells (9, 10) were used in ADME studies.

DS®3.0 selects drug-like ligands according to rules formulated by Lipinski (RO5) (14, 15). The parameters for the ligands that pass the filter and for the ligands that failed to pass the filter are presented in Table 2 and Table 3, respectively.

ADME properties of compounds **1**-**12** have been calculated and the results are presented in Tables 4 and 5. All calculations were solely based on the chemical structure of molecules.

The graphical illustration of the human intestinal absorption model (HIA) and blood brain barrier penetration model (BBB) is the ADME 2D Plot (Fig. 1) drawn using ADMET_PSA_2D and ADMET_AlogP98 properties calculated for all the tested compounds. The HIA model includes 95% and 99% confidence ellipses which define regions where well-absorbed compounds are expected to be found. Ninety five percent and 99% confidence ellipsoids corresponding to blood brain penetration model are not the same as those associated with HIA model, although they have an analogous interpretation (13).

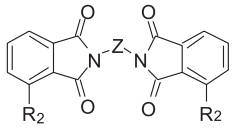
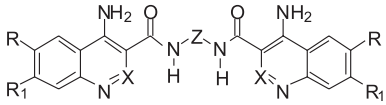
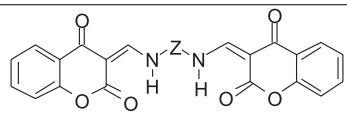
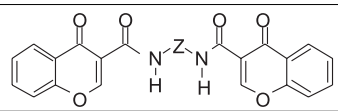
DISCUSSION

Lipinski Rule of five (RO5) and Veber rule

In our effort to design potential anticancer drugs we obtained 36 polyamine derivatives **1**-**12** containing dimeric phthalimide **1**, **2,** quinoline **3-6**, cinnoline **7-10**, and chromone **11, 12** moieties. The synthetic route and biological activity of selected compounds have been already published (9, 10). In the present study, all the compounds were subjected to *in silico* ADME screening by evaluating their theoretical drug-likeness and physicochemical properties using Discovery Studio 3.0 obtained from Accelrys.

Among the tested compounds, 8 molecules **1a**, **1c**, **2c**, **3c**, **11a**, **11c**, **12a**, **12c** showed drug-like characteristics based on Lipinski and Veber rules (Table 2), which meant that they had a higher probability of good absorption after oral dosing. The largest proportion of drug-like compounds was among dimeric chromone (4 / 6) and phthalimide (3 / 6) derivatives. Within these two groups, molecules with terminal moieties tethered by 1,4-bis(3-aminopropyl)piperazine (**a**) or 3,3'-diamino-*N*-methyldipropylamine (**c**) were privileged. It is well worth noting that **11c**

Table 1. Polyamine derivatives evaluated by ADME *in silico* screening.

Comp. no.		Z	X	R	R_1	R_2
1a		a	-	-	-	-
1b		b	-	-	-	-
1c		c	-	-	-	-
2a		a	-	-	-	NO_2
2b		b	-	-	-	NO_2
2c		c	-	-	-	NO_2
3a		a	CH	-	-	-
3b		b	CH	-	-	-
3c		c	CH	-	-	-
4a		a	CH	CH_3	-	-
4b		b	CH	CH_3	-	-
4c		c	CH	CH_3	-	-
5a		a	CH	-	CH_3	-
5b		b	CH	-	CH_3	-
5c		c	CH	-	CH_3	-
6a		a	CH	CH_3	CH_3	-
6b		b	CH	CH_3	CH_3	-
6c		c	CH	CH_3	CH_3	-
7a		a	N	-	-	-
7b		b	N	-	-	-
7c		c	N	-	-	-
8a		a	N	CH_3	-	-
8b		b	N	CH_3	-	-
8c		c	N	CH_3	-	-
9a		a	N	-	CH_3	-
9b		b	N	-	CH_3	-
9c		c	N	-	CH_3	-
10a		a	N	CH_3	CH_3	-
10b		b	N	CH_3	CH_3	-
10c		c	N	CH_3	CH_3	-
11a		a	-	-	-	-
11b		b	-	-	-	-
11c		c	-	-	-	-
12a		a	-	-	-	-
12b		b	-	-	-	-
12c		c	-	-	-	-

a – $-(CH_2)_3N(CH_2CH_2)_2N(CH_2)_3-$, b – $-(CH_2)_3O(CH_2)_4O(CH_2)_3-$, c – $-(CH_2)_3NCH_3(CH_2)_3-$

was active at inhibition of both cell proliferation and adhesive potential of A375 melanoma cells (10). In addition, dimeric phthalimide **1b** and chromone **11b**, **12b** derivatives containing 4,9-dioxa-1,12-dodecanediamine (**b**) as a spacer, have failed to pass only the Veber rule filter due to the rotatable bond count exceeding 10, therefore, according to Lipinski's rule they exhibited drug-like properties.

As far as dimeric quinoline derivatives **3**-**6** were concerned, most of them were found to violate

Table 2. RO5 and Veber Rules parameters for the ligands that pass the filter.

Comp. no.	Lipinski Rule				Veber Rule			
	HBD*	HBA*	M.w.	Alog*P*	RB	Molecular_PSA	HBA	HBD
1a	0	8	460.5	1.764	8	81.23	6	0
1c	0	7	405.4	1.806	8	78.00	5	0
2c	0	13	495.4	1.595	10	169.64	9	0
3c	6	9	485.6	1.037	10	139.26	7	4
11a	2	10	544.6	1.152	10	117.28	8	2
11c	2	9	489.5	1.194	10	114.04	7	2
12a	2	10	544.6	1.541	10	117.28	10	2
12c	2	9	489.5	1.583	10	114.04	9	2

HBD – number of hydrogen bond donors (*according to Lipinski rule), HBA – number of hydrogen bond acceptors (*according to Lipinski rule), RB – number of rotatable bonds, M.w. – molecular weight, Alog*P* – the log value of octanol-water partition coefficient, Molecular_PSA – molecular polar surface area.

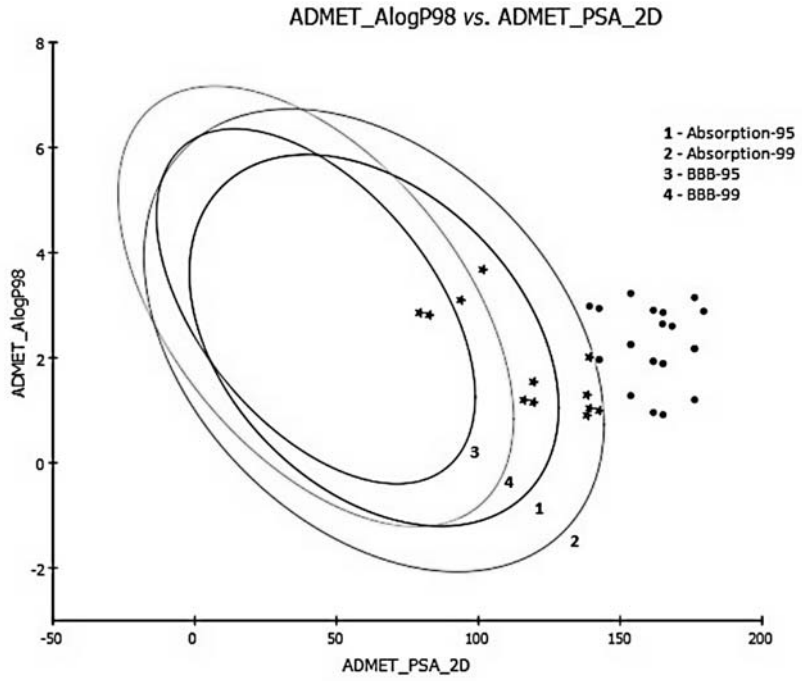


Figure 1. Plot of polar surface area (PSA) *vs.* LogP for tested compounds showing the 95% and 99% confidence limits ellipses corresponding to the blood brain barrier and intestinal absorption models

RO5 mainly due to an Mw > 500 and H-bond donors > 5. Additionally, quinoline derivatives **3b**, **4b**, **5b**, **6b** like other compounds with 4,9-dioxa-1,12-dodecanediamine (**b**) as a linker, broke the Veber Rule by number of rotatable bonds > 10 (Table 3). Only one agent containing quinoline moieties **3c** was selected as a drug-like molecule because its structure was in accordance with both Lipinski and Veber rules (Table 2) but none of cinnoline compounds exhibited drug-like characteristics. They violated the RO5 by Mw > 500, H-bond donors >5, H-bond acceptors > 10, and the Veber Rule by PSA >140 and the sum of HBD and HBA > 12. In case of compounds **8b**, **9b** and **10b** containing 4,9-dioxa-1,12-dodecanediamine (**b**) as the linker, number of rotatable bonds exceeded 10 as well.

Table 3. RO5 and Veber Rules parameters for the ligands that failed to pass the filter.

Comp. no.	Lipinski Rule				Veber Rule				Fail reason
	HBD*	HBA*	M.w.	AlogP	Molecular_PSA	RB	HBA	HBD	
1b	0	8	464.51	2.048	93.22	6	0	13	RB > 10
2a	0	14	550.52	1.553	172.88	10	0	10	HBA > 10; Mw > 500
2b	0	14	554.50	1.837	184.86	10	0	15	HBA > 10; Mw > 500; RB > 10
3a	6	10	540.66	0.995	142.5	10	8	4	HBD > 5; Mw > 500
3b	6	10	544.64	1.279	154.48	15	8	4	HBD >5; Mw > 500; RB > 10
4a	6	10	568.71	1.967	142.5	10	8	4	HBD > 5; Mw > 500
4b	6	10	572.69	2.251	154.48	15	8	4	HBD > 5; Mw > 500; RB > 10;
4c	6	9	513.63	2.01	139.26	10	7	4	HBD > 5; Mw > 500;
5a	6	10	568.71	1.967	142.5	10	8	4	HBD > 5; Mw > 500
5b	6	10	572.69	2.251	154.48	15	8	4	HBD > 5; Mw > 500; RB > 10
5c	6	9	513.63	2.01	139.26	10	7	4	HBD >5; Mw > 500;
6a	6	10	596.77	2.939	142.5	10	8	4	HBD > 5; Mw > 500
6b	6	10	600.75	3.224	154.48	15	8	4	HBD > 5; Mw > 500; RB > 10
6c	6	9	541.69	2.982	139.26	10	7	4	HBD > 5; Mw > 500
7a	6	12	542.63	0.917	168.28	10	10	4	HBD >5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12
7b	6	12	546.62	1.201	180.26	15	10	4	HBD > 5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12
7c	6	11	487.55	0.959	165.04	10	9	4	HBD > 5; HBA> 10; PSA >140; HBD + HBA > 12
8a	6	12	570.68	1.889	168.28	10	10	4	HBD > 5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12

Table 3. Cont.

Comp. no.	Lipinski Rule				Veber Rule				Fail reason
	HBD*	HBA*	M.w.	AlogP	Molecular_PSA	RB	HBA	HBD	
8b	6	12	574.67	2.174	180.26	15	10	4	HBD > 5; HBA > 10; Mw > 500; RB > 10; PSA > 140; HBD + HBA > 12;
8c	6	11	515.61	1.932	165.04	10	9	4	HBD > 5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12
9a	6	12	570.68	1.889	168.28	10	10	4	HBD > 5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12
9b	6	12	574.67	2.174	180.26	15	10	4	HBD > 5; HBA > 10; Mw > 500; RB > 10; PSA > 140; HBD + HBA > 12
9c	6	11	515.61	1.932	165.04	10	9	4	HBD > 5; HBA> 10; Mw > 500; PSA > 140; HBD + HBA > 12
10a	6	12	598.74	2.862	168.28	10	10	4	HBD > 5; HBA > 10; Mw > 500; PSA > 140; HBD + HBA > 12
10b	6	12	602.73	3.146	180.26	15	10	4	HBD > 5; HBA > 10; Mw > 500, RB > 10, PSA > 140, HBD + HBA > 12
10c	6	11	543.66	2.904	165.04	10	9	4	HBD>5; HBA> 10; Mw> 500; PSA> 140; HBD + HBA > 12
11b	4	10	546.61	1.293	134.85	10	4	15	RB > 10
12b	4	10	546.61	0.904	134.86	8	4	15	RB > 10

HBD - number of hydrogen bond donors (*according to the Lipinski rule), HBA - number of hydrogen bond acceptors (*according to the Lipinski rule), RB - number of rotatable bonds, M.w. - molecular weight, AlogP - the log value of octanol-water partition coefficient, Molecular_PSA - molecular polar surface area.

ADME properties prediction

Proper ADME profile of drug candidates is crucial factor determining its entry to the market. It is of great importance to start evaluating pharmacokinetic profile as early as possible in drug discovery and development process in order to avoid late stage attrition.

ADMET_solubility

According to ADMET_solubility model in DS®3.0, the solubility of tested compounds varied from low to optimal. From Table 4, it is clear that the aqueous solubility of all dimeric phthalimide **1**, **2** and quinoline **3**-**6** derivatives except for **3b** and **6c**, was good. In case of dimeric cinnoline molecules, aqueous

Table 4. ADME parameters calculated for designed compounds.

Comp. no.	ADMET Solubility logS_w	ADMET Solubility level	ADMET Absorption level	ADMET Alog*P*98	ADMET PSA 2D	ADMET BBB	ADMET BBB level
1a	-3.581	3	0	2.813	82.613	-0.592	3
1b	-2.851	3	0	3.097	93.768	-0.68	3
1c	-3.782	3	0	2.855	79.260	-0.526	3
2a	-2.959	3	3	2.602	168.259	-	4
2b	-2.120	3	3	2.886	179.414	-	4
2c	-3.422	3	3	2.644	164.906	-	4
3a	-2.730	3	1	0.995	142.529	-	4
3b	-1.881	4	3	1.279	153.684	-	4
3c	-2.732	3	1	1.037	139.176	-	4
4a	-3.337	3	2	1.967	142.529	-	4
4b	-2.448	3	3	2.252	153.684	-	4
4c	-3.441	3	1	2.010	139.176	-	4
5a	-3.306	3	2	1.967	142.529	-	4
5b	-2.417	3	3	2.252	153.684	-	4
5c	-3.415	3	1	2.010	139.176	-	4
6a	-3.885	3	2	2.939	142.529	-	4
6b	-2.954	3	3	3.224	153.684	-	4
6c	-4.099	2	2	2.982	139.176	-	4
7a	-3.631	3	3	0.917	165.050	-	4
7b	-2.783	3	3	1.201	176.206	-	4
7c	-3.633	3	3	0.959	161.698	-	4
8a	-4.239	2	3	1.889	165.050	-	4
8b	-3.350	3	3	2.174	176.206	-	4
8c	-4.343	2	3	1.932	161.698	-	4
9a	-4.208	2	3	1.889	165.050	-	4
9b	-3.319	3	3	2.174	176.206	-	4
9c	-4.316	2	3	1.932	161.698	-	4
10a	-4.787	2	3	2.862	165.050	-	4
10b	-3.856	3	3	3.146	176.206	-	4
10c	-5.000	2	3	2.904	161.698	-	4
11a	-1.672	4	0	1.152	119.388	-	4
11b	-1.276	4	1	0.904	138.304	-	4
11c	-2.021	3	0	1.194	116.036	-	4
12a	-2.157	3	0	1.541	119.388	-	4
12b	-1.761	4	1	1.293	138.304	-	4
12c	-2.514	3	0	1.583	116.036	-	4

ADMET Solubility level (categorical solubility level: 0 – extremely low, 1 – very low but possible, 2 – low, 3 – good, 4 – optimal, 5 – too soluble, 6 – warning: molecules with one or more unknown AlogP98 types (16), ADMET Solubility logS_w – the base 10 logarithm of the molar solubility as predicted by the regression. ADMET Absorption level (categorical absorption level: 0 – good absorption, 1 – moderate absorption, 2 – poor absorption, 3 – very poor absorption (17, 18), ADMET BBB – the base 10 logarithm of (brain concentration)/(blood concentration) as predicted by a robust (least-median-of-squares) regression derived from literature *in vivo* brain penetration data. ADMET BBB level (categorical level: 0 – very high penetrant, 1 – high penetrant, 2 – medium penetrant, 3 – low penetrant 4 – undefined (13, 18).

Table 5. ADME parameters calculated for designed compounds.

Comp. no.	ADME EXT CYP2D6	ADME EXT CYP2D6#Prediction	ADME EXT HEPATOTOXIC	ADME EXT Hepatotoxic#Prediction	ADMET EXT PPB	ADMET EXT PPB#Prediction
1a	-0.933	false	-7.154	false	2.649	true
1b	-2.023	false	-5.830	false	-0.335	true
1c	-1.162	false	-5.544	false	-0.872	true
2a	-4.145	false	-3.899	true	1.123	true
2b	-5.677	false	-2.575	true	-2.270	false
2c	-4.868	false	-2.521	true	-2.926	false
3a	-3.426	false	-1.449	true	-1.999	true
3b	-4.957	false	-0.529	true	-5.058	false
3c	-4.149	false	-0.071	true	-5.507	false
4a	-2.969	false	-3.746	true	-1.052	true
4b	-3.808	false	-2.855	true	-3.641	false
4c	-3.692	false	-2.397	true	-4.318	false
5a	-3.203	false	-2.868	true	-0.157	true
5b	-4.041	false	-1.977	true	-2.746	false
5c	-3.926	false	-1.519	true	-3.423	false
6a	-1.411	false	-2.180	true	-0.088	true
6b	-3.402	false	-1.394	true	-2.836	false
6c	-2.593	false	-0.936	true	-3.236	false
7a	-5.084	false	-2.328	true	-0.076	true
7b	-6.615	false	-1.407	true	-3.493	false
7c	-5.807	false	-0.949	true	-3.803	false
8a	-4.497	false	-4.344	false	0.856	true
8b	-5.335	false	-3.192	true	-1.614	true
8c	-5.166	false	-2.734	true	-2.511	false
9a	-4.731	false	-3.870	true	1.323	true
9b	-5.569	false	-2.717	true	-1.147	true
9c	-5.399	false	-2.259	true	-2.044	true
10a	-3.292	false	-3.182	true	1.3160	true
10b	-4.929	false	-2.395	true	-1.356	true
10c	-4.015	false	-1.803	true	-1.975	true
11a	-2.974	false	-0.306	true	2.987	true
11b	-4.049	false	2.610	true	-1.345	true
11c	-4.156	false	1.073	true	-0.520	true
12a	-0.139	false	-0.123	true	0.04380	true
12b	-2.704	false	2.926	true	-2.910	false
12c	-1.713	false	1.255	true	-2.411	false

ADMET EXT CYP2D6 – Bayesian score from the model (19), ADMET EXT Hepatotoxic – Bayesian score from the model (19, 20), ADMET EXT PPB – Bayesian score from the model (19).

solubility of **8a**, **8c**, **9a**, **9c**, **10a**, **10c** was predicted to be low in contrast to **7a-c**, **8b**, **9b**, **10b** which exhibited good soluble character. Results presented in Table 4 suggest an optimal aqueous solubility for dimeric chromone **12a**, **12b** and **11b** and quinoline **3b** derivatives containing 1,4-bis(3-aminopropyl)piperazine (**a**) and 4,9-dioxa-1,12-dodecanediamine (**b**) as spacers. It made them promising entities for further evaluation.

ADMET_Human Intestinal Absorption (HIA)

Analyzing the ADME plot (Fig. 1), it could be observed that polyamine derivatives containing dimeric phthalimide **1a-c**, and chromone moieties **11a**, **11c**, **12a**, **12c** were fallen inside the 99% absorption ellipse, so they were expected to possess good human intestinal absorption (HIA) when compared to other compounds. HIA of dimeric quinoline derivatives were predicted as moderate **3a**, **3c**, **4c**, **5c**, poor **4a**, **5a**, **6a**, **6c** or very poor for compounds with 4,9-dioxa-1,12-dodecanediamine (**b**) as the linker **3b** and **6b** (Table 4). It was also predicted that dimeric cinnoline derivatives **7**-**10** could not be transported across the intestinal ephitelium as they all have fallen outside the 99% absorption ellipse in ADME plot (Fig. 1).

ADMET_Blood Brain Barrier

In ADME plot (Fig. 1) almost all examined compounds: dimeric phthalimide **2** quinoline **3**-**6**, cinnoline **7**-**10** and chromone **11**, **12** derivatives were fallen outside 99% BBB confidence ellipse which meant the quality of the results were unknowable (undefined level = 4). Only dimeric phthalimide derivatives **1** possessed low penetration capacity (brain-blood ratio less than 0.3 : 1). Such a blood brain barrier penetration level (Table 4) might indicate that possible central nervous system side effects would be low or absent, but it would be limiting factor in the possible therapy of brain tumors.

ADMET_Cytochrome P450 2D6 (CYP2D6)

The cytochrome P450 2D6 (CYP2D6) model predicts CYP2D6 enzyme inhibition (19). CYP2D6 is involved in the metabolism of a wide range of xenobiotics and its inhibition by a drug may lead to serious drug-drug interactions. Therefore, CYP2D6 inhibition experiment is indispensable as part of the drug discovery and development process (5). All of the examined compounds **1**-**12** were classified as non-inhibitors of CYP2D6 (Table 5) using the cut-off Bayesian score of 0.162 (13, 19). Hence, potential adverse effects resulting from drug interactions upon oral administration of these compounds are unlikely.

ADMET_Hepatotoxicity

The hepatotoxicity model predicts the incidence of dose dependent human toxicity. Compounds are classified as hepatotoxic or non-hepatotoxic using the cut-off Bayesian score of -0.4095 (obtained by minimizing the total number of false positives and false negatives) (13, 19). According to the DS®3.0 Hepatotoxicity model almost all of examined compounds (Table 5) were classified as hepatotoxic, which may be the major obstacle to further evaluation. Only four derivatives, dimeric phthalimide **1a-c** and cinnoline **8a** derivatives were classified as non-toxic.

ADMET_Plasma Protein Binding

The pharmaceutical activity is determined by only the free drug concentration, therefore possible interaction of compounds with plasma protein binding has to be taken into account during discovery process (21). The plasma protein binding model allows predicting, if a compound is likely to be highly bound (= 90% bound) to plasma proteins. The classification whether a compound is highly bounded (= 90% bound) to plasma proteins is based on the cut-off Bayesian score of -2.226 (22). The results disclosed in Table 5 showed that 21 of the 36 tested compounds **1a-c**, **2a**, **3a-6a 7a**, **8a**, **8b**, **9a-c**, **10a-c**,**12a-c** and **11a** were likely to be highly bound to the plasma proteins. Molecules with1,4-bis(3-aminopropyl)piperazine (**a**) as the linker predominated in this group.

CONCLUSION

Evaluating the pharmacokinetic profile of our polyamine derivatives **1**-**12** we particularly focused on molecules having anti-proliferative activity against A375 melanoma cell line: **3a**, **3b** and **11c**. Dimeric quinoline derivatives were characterized by good **3a** and very good **3b** aqueous solubility but they were classified as moderately, or very poorly absorbed after oral administration. In case of dimeric 2*H*-chromeno-2,4(3*H*)-dione derivative **11c**, both aqueous solubility level and absorption level were predicted as good. Selected compounds were found as non-inhibitors of CYP2D6 but were likely to be hepatotoxic. Only compound **3b** was defined to possess high affinity toward plasma proteins.

Taking into account computer aided ADME studies of compounds which exhibited antiproliferative activity against melanoma cells, it can be concluded that they met the requirements of potential drugs for their pharmacokinetic profile with the exception of hepatotoxicity, which may be the main obstacle to further development. Apart from hepatotoxicity, drug-like characteristics of **3a** and **3b** need to be improved. In addition, **11c** exhibiting the most promising activity *in vitro* (10) showed favorable drug-like properties according to the Lipinski and the Veber Rules, which makes it valuable lead candidate.

Considering other compounds, phthalimide derivatives **1a-c** met the required ADME criteria:

good solubility and intestinal absorption, lack of CYP2D6 inhibition or hepatotoxicity. They also exhibited drug-like characteristics according to RO5. Although they demonstrated no inhibitory activities against melanoma cells it would be of interest to evaluate their biological activity on other cell lines. In case of cinnoline derivatives, lack of antiproliferative activity and poor pharmacokinetic profile indicate that this group of compounds should be no longer evaluated.

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SYNTHESIS AND IMMUNOREGULATORY PROPERTIES OF SELECTED 5-AMINO-3-METHYL-4-ISOXAZOLECARBOXYLIC ACID BENZYLAMIDES

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Abstract: The aim of the study was to characterize a series of isoxazole derivatives in several immunological tests *in vitro* and *in vivo*, in mouse and human models. The human model included measurement of: viability of peripheral blood mononuclear cells (PBMC), phytohemagglutinin A (PHA)-induced proliferation of PBMC, production of tumor necrosis factor α (TNF α) in whole blood cultures stimulated with lipopolysaccharide (LPS) and growth of SW-948 and L1210 tumor cell lines. Experiments in mice encompassed the following tests: secondary, humoral immune response splenocytes to sheep erythrocytes (SRBC) *in vitro*, delayed type hypersensitivity (DTH) to ovalbumin (OVA) and carrageenan-induced foot edema. All compounds were non-toxic against PMBC and displayed differential, dose-dependent suppressive properties in the model of PHA-induced PMBC proliferation. They also exhibited differential, mostly inhibitory effects on TNF α production. The inhibitory actions on growth of tumor cell lines were moderate. **MO5** (5-amino-3-methyl-N-(4-methylbenzyl)-4-isoxazolecarboxamide) was most suppressive in the proliferation and TNF α production tests, it was, therefore, selected for *in vitro* and *in vivo* studies in the mouse models. The compound inhibited the humoral immune response *in vitro*, stimulated the inductive phase of DTH *in vivo*, although it inhibited the eliciting phase of that response. The compound also inhibited the carrageenan skin reaction. **MO5** combines strong anti-proliferative and anti-inflammatory activities, it is therefore attractive for further studies in more advanced animal models as a potential therapeutic.

Keywords: carrageenan; immune response; isoxazoles; PBMC; PHA; TNF α

The hitherto efforts, aimed to supply for the pharmaceutical market new therapeutics that would prove effective in treatment of diseases resulting from overstimulation of suppression of the immune system, have been insufficient. Therefore, extensive search for new compounds from various classes of chemicals is continued. The isoxazole system has been a source of valuable drugs. Isoxazoles belong to the most important azoles (1). A number of pharmaceuticals as well as bioactive natural products integrate azoles, and specifically isoxazoles, as key pharmacophores pivotal for biological activities of drugs such as: valdecoxib (COX-2 inhibitor), sulfamethoxazole (PABA antagonist), oxacillin (β-lactam antibiotic), isoxaflutole (4-hydroxyphenylpyruvate dioxygenase inhibitor) or leflunomide (anti-rheumatic drug) (2). In these molecules, isoxazole acts as an indispensable pharmacophore enforcing the desired pharmacological activity due to a unique positioning in a 1,2-relationship of two electronegative heteroatoms that are capable of engaging in hydrogen bond donor/acceptor interactions with a variety of enzymes, receptors and messengers, unavailable for other rings systems and potential therapeutics (3, 4).

Isoxazole derivatives were reported to possess a variety of biological activities including antidepressant (5), dopamine D 4 receptor activity (6), anticonvulsant (7), antiviral (8), and antifungal (9). In our studies, (10-12) isoxazole derivatives with immunostimulatory and immunosuppressive properties were found.

We obtained the mono-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides (Fig. 1) with the immunostimulatory activity equal to or higher than levamisole (a reference drug).

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Further characterization of these compounds showed their very low toxicity (10-12).

Among these compounds, 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides, substituted in position 4 with a strong electrophilic group, were most active. 4-Chlorophenylamide of 5-amino-3-methyl-4-isoxazolecarboxylic acid (Fig. 2) proved strong stimulating effect on the humoral and cellular immune responses (10-12).

In contrast, di- and tri-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides showed immunosuppressive activity more effective than cyclosporine A (a reference drug). Dichloro- and trimethoxy- derivatives were inhibitors of the humoral immune response (11-13).

Also, N'-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide (Fig. 3) showed immunosuppressive activity in mouse and human experimental models (14).

An interesting immunomodulatory activity of the compounds described above led us to synthesize a new series of 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides substituted on the phenyl ring (Fig. 4)

Then, biological and quantum-chemical studies for obtained compounds were performed. The

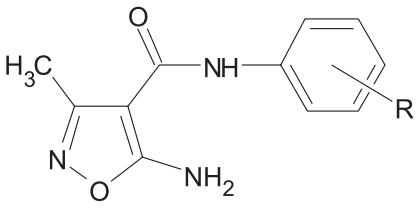


Figure 1. Mono-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid phenylamides

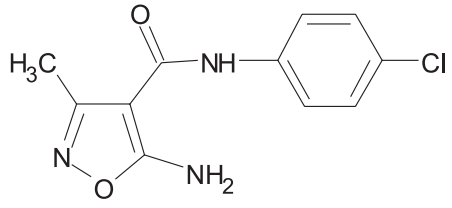


Figure 2. 4-Chlorophenylamide of 5-amino-3-methyl-4-isoxazolecarboxylic acid

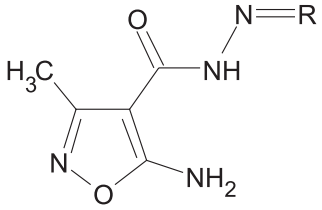


Figure 3. N'-substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid hydrazide

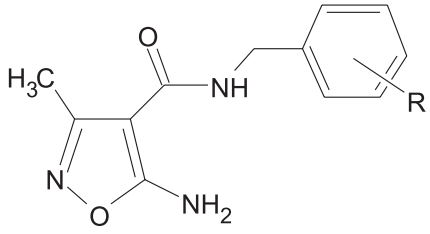


Figure 4. 5-Amino-3-methyl-4-isoxazolecarboxylic acid benzylamides substituted on the phenyl ring

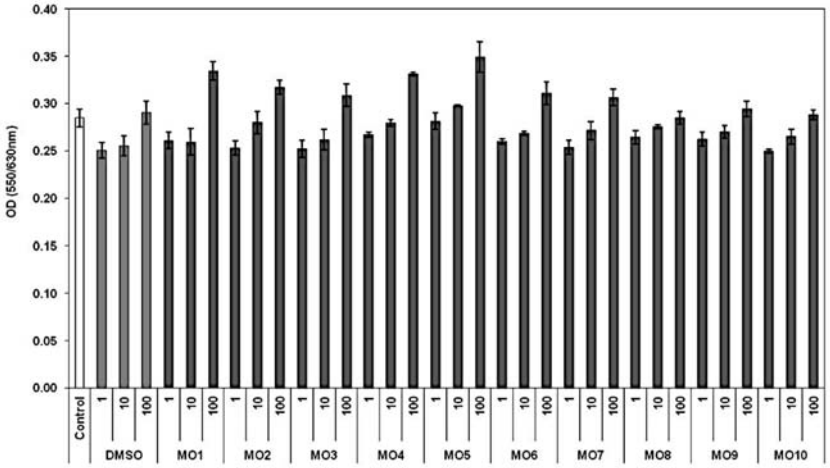


Figure 5. Toxicity of the compounds against human PBMC. The compounds were used at concentrations of 1, 10 and 100 μg/mL. The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations. The cell toxicity was compared with toxicity of DMSO used as solvent

results led to evaluation of the immunological activities and the structure-activity relationships of this class of compounds.

The aim of this study was to synthesize substituted 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides, evaluate their immunological properties in preliminary models *in vitro*, select a most active compound and study its activity in the mouse models on nonspecific and specific immune responses *in vivo*.

EXPERIMENTAL

Biology

Animals

CBA mice of both sexes, 8-12 week old, derived from a breeding colony in Ilkowice, Poland were used. The mice were kept in standard conditions with free access to granulated food and filtered water. The local ethics committee for animal experiments approved the study (Institute of Immunology and Experimental Therapy, Polish Academy of Sciences in Wrocław, 20/01/2010, 8/2010).

Reagents

Hanks' medium, RPMI-1640 medium, Eagle's medium and LSM (lymphocyte separation medium; 1.077 g/mL) were from Cytogen, Sinn, Germany; MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), L-glutamine, sodium pyruvate, 2-mercaptoethanol, 0.83% NH_4Cl solution, antibiotics (penicillin and streptomycin), lipopolysaccharide (LPS) from *Escherichia coli,* serotype O:111:B4, concanavalin A (ConA), cyclosporine A (CsA), cisplatin and carrageenan were from Sigma, Saint Louis, U.S.A. Freund's complete adjuvant (cFa), Freund's incomplete adjuvant (iFa) and fetal calf serum (FCS) were from BD Biosciences, U.S.A.. Dexamethasone (Dexaven®) was from Jelfa, Poland. Sheep red blood cells (SRBC) were supplied by Wrocław University of Life and Environmental Sciences, Poland. TNF α was measured by enzyme-linked immunoassay (ELISA Ready-SET-Go Affymetrix eBioscience). Cyclooxygenase (COX) Inhibitor Screening Assay Kit was supplied by Cayman Chemical, Ann Arbor, U.S.A.

Preparation of the compounds for biological assays

The compounds were dissolved in DMSO (5 mg/200 µL) and subsequently diluted to 1 mL of RPMI-1640 medium for *in vitro* studies. As a control, appropriate dilutions of DMSO in RMPI-1640 medium were used. For *in vivo* administration the compound was further diluted in 0.9% saline.

Isolation of the human peripheral blood mononuclear cells (PBMC)

Venous peripheral blood from a single donor (healthy adult volunteer) was withdrawn into heparinized syringes and diluted twice with PBS. PBMC were isolated by centrifugation on lymphocyte separation medium and centrifuged at 400 × g for 20 min at 4°C. The interphase cells were then washed three times with Hanks' medium and resuspended in a culture medium, referred to below as the culture medium, consisting of RPMI-1640, supplemented with 10% FCS, L-glutamine, sodium pyruvate, 2-mercaptoethanol and antibiotics, at density of 2×10^6 cells/mL.

Cytotoxicity of the compounds against human PBMC

PBMC at density of 3×10^5/100 µL/well, resuspended in the culture medium, were cultured for 24 h in a cell culture incubator with the compounds at 1, 10 and 100 µg/mL concentrations. Cell survival was determined by MTT colorimetric method (15). The data are presented as a mean optical density (OD) values at 550 nm ± standard error (SE) from quadruplicate determinations.

Phytohemagglutinin A (PHA)-induced proliferation of human blood mononuclear cells

PBMC were distributed into 96-well flat-bottom plates in 100 µL aliquots (2×10^5 cells/well). PHA was added at a concentration of 5 µg/mL. The compounds were tested at doses of 1, 10 and 100 µg/mL. DMSO at appropriate dilutions served as control. After a four-day incubation in a cell culture incubator, the proliferative response of the cells was determined by the MTT colorimetric method (15). The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations.

Lipopolysaccharide (LPS)-induced TNF α production in whole blood cell culture

Venous blood from a single donor (healthy adult volunteer) was diluted 10 × with RPMI-1640 medium and distributed in 1 mL aliquots in 24-well culture plates. The cultures were stimulated by addition of 1 µg/mL of LPS. The compounds were added to the cultures at concentrations of 1-100 µg/mL**.** Appropriate dilutions of DMSO served as controls. After overnight incubation in a cell culture incubator, the supernatants were harvested and frozen at -20°C until cytokine determination by ELISA kit

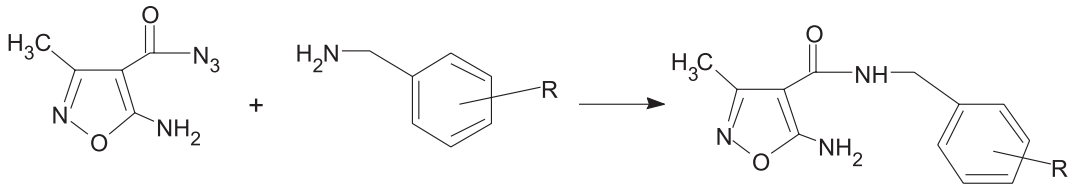
according to manufacturer's instructions. The results are presented in pg/mL.

Tumor cell lines

L-1210 lymphoma and SW-948 colon tumor cell lines derived from the Collection of Cell Lines of The Institute of Immunology and Experimental Therapy, Wrocław, Poland. The lines were resuspended in the culture medium and distributed into 96-well flat bottom plates. L-1210 was present at 1.5×10^4 cells/100 µL/well while SW-948 at 2.5×10^4 cells/100 µL/well. Cisplatin served as a reference drug. The studied preparations were added to the wells at the indicated concentrations. After 3-day incubation in a cell culture incubator, the proliferation was determined using MTT colorimetric method (15). The data are presented as mean OD values at 550 nm ± SE from quadruplicate determinations.

Secondary humoral immune response in vitro

Mice (n = 5) were sensitized intraperitoneally (*i.p.*) with 0.2 mL of 5% sheep red blood cells (SRBC) suspension. After 4 days, the spleens were isolated, a single cell suspension prepared by pressing the organs against a plastic screen into a cold Hanks' medium, washed 2× with Hanks' medium and resuspended in the culture medium at a density of 5×10^6/mL. The cells were distributed to 24-well culture plates in 1 mL aliquots and 0.05 mL of 0.005% SRBC was added as antigen. MO5 compound was used at 1, 10 and 100 µg/mL. The number of antibody forming cells (AFC) in the cultures



Scheme 1. Synthesis of 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides (MO series)

Table 1. The analytical data of the obtained compounds.

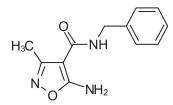
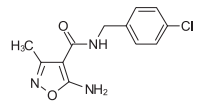
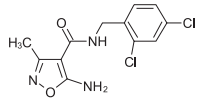
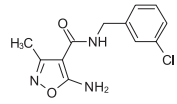
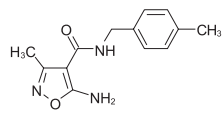
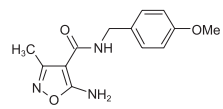
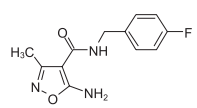
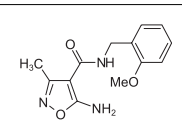
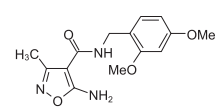
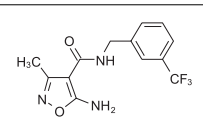
Compound	Melting point (°C)	Yield (%)	Formula Molecular weight (g/mol)
MO1	126.5-127.5	45	$C_{12}H_{13}N_3O_2$ 231.25
MO2	146-147	52	$C_{12}H_{12}ClN_3O_2$ 265.70
MO3	167-168	54	$C_{12}H_{11}Cl_2N_3O_2$ 300.15
MO4	133-134	48	$C_{12}H_{11}ClN_3O_2$ 265.70
MO5	152-153	63	$C_{13}H_{15}N_3O_2$ 245.28
MO6	145-146	58	$C_{13}H_{15}N_3O_3$ 261.28
MO7	176-177	49	$C_{12}H_{12}FN_3O_2$ 249.24
MO8	167-168	64	$C_{13}H_{15}N_3O_3$ 261.28
MO9	162-163	57	$C_{14}H_{17}N_3O_4$ 291.30
MO10	187-188	52	$C_{13}H_{12}F_3N_3O_2$ 299.25

were determined using a method of local hemolysis in agar gel (16). The results are presented as a mean numbers of AFC per 10^6 splenocytes ± SE.

Delayed type hypersensitivity to ovalbumin (OVA)

Mice (n = 5) were sensitized subcutaneously (*s.c.*) with 5 µg ovalbumin (OVA) emulsified in Freund's complete adjuvant (cFa) in the tail base. After 4 days, the mice were challenged *s.c.* with 50 µg OVA in incomplete Freund's antigen (iFa) in the hind footpads. Following next 24 h, the footpad thickness was measured using a spring caliper with 0.05 mm accuracy. **MO5** compound was administered to mice *i.p.*, at 100 µg dose, 30 min before sen-

Table 2. The structure and spectroscopic data of the obtained compounds.

Compound	Chemical structure	Spectroscopic data
MO1		^{1}H NMR (DMSO-d_6, δ, ppm): 2.288 (s, 3H, CH_3), 4.381-4.401 (d, 2H, CH_2), 7.221-7.300 (m, 5H, CH-aromat), 7.415 (t, 1H, NH), 7.455 (s, 2H, NH_2). IR (cm^{-1}): 1659 C=O, 3324 NH, 3428-3441 NH_2
MO2		^{1}H NMR (DMSO-d_6, δ, ppm): 2.282 (s, 3H, CH_3), 4.350-4.370 (d, 2H, CH_2), 7.292-7.320 (d, 2H, CH-aromat), 7.343-7.372 (d, 2H, CH-aromat), 7.468 (t, 1H, NH), 7.478 (s, 2H, NH_2). IR (cm^{-1}): 1652 C=O, 3324 NH, 3424-3446 NH_2
MO3		^{1}H NMR (DMSO-d_6, δ, ppm): 2.310 (s, 3H, CH_3), 4.404-4.423 (d, 2H, CH_2), 7.322-7.408 (m, 2H, CH-aromat), 7.415 (s, 1H, CH-aromat), 7.434-7.496 (t, 1H, NH), 7.579 (s, 2H, NH_2). IR (cm^{-1}): 1658 C=O, 3316 NH, 3417-3442 NH_2
MO4		^{1}H NMR (DMSO-d_6, δ, ppm): 2.290 (s, 3H, CH_3), 4.366-4.386 (d, 2H, CH_2), 7.270-7.338 (m, 3H, CH-aromat), 7.363 (s, 1H, CH-aromat), 7.444 (t, 1H, NH), 7.478 (s, 2H, NH_2). IR (cm^{-1}): 1660 C=O, 3322 NH, 3420-3445 NH_2
MO5		^{1}H NMR (DMSO-d_6, δ, ppm): 2.317 (s, 3H, CH_3), 2.343 (s, 3H, CH_3), 4.522-4.541 (d, 2H, CH_2), 7.085-7.112 (d, 2H, CH-aromat), 7,161-7,188 (d, 2H, CH-aromat), 7.304-7.376 (t, 1H, NH), 7.452 (s, 2H, NH_2). IR (cm^{-1}): 1657 C=O, 3319 NH, 3421-3443 NH_2
MO6		^{1}H NMR (DMSO-d_6, δ, ppm): 2.268 (s, 3H, CH_3), 3.705 (s, 3H, OCH_3), 4.301-4.321 (d, 2H, CH_2), 6.843-6.872 (d, 2H, CH-aromat), 7.202-7.231 (d, 2H, CH-aromat), 7.319-7.359 (t, 1H, NH), 7.445 (s, 2H, NH_2). IR (cm^{-1}): 1655 C=O, 3315 NH, 3416-3439 NH_2
MO7		^{1}H NMR (DMSO-d_6, δ, ppm): 2.280 (s, 3H, CH_3), 4.350-4.369 (d, 2H, CH_2), 7.117-7.146 (d, 2H, CH-aromat), 7.302-7.321 (d, 2H, CH-aromat), 7.349-7.413 (t, 1H, NH), 7.458 (s, 2H, NH_2). IR (cm^{-1}): 1661 C=O, 3322 NH, 3424-3447 NH_2
MO8		^{1}H NMR (DMSO-d_6, δ, ppm): 2.299 (s, 3H, CH_3), 3.811 (s, 3H, OCH_3), 4.359-4.379 (d, 2H, CH_2), 6.866-7.166 (m, 4H, CH-aromat), 7.183-7.235 (t, 1H, NH), 7.448 (s, 2H, NH_2). IR (cm^{-1}): 1657 C=O, 3323 NH, 3418-3443 NH_2
MO9		^{1}H NMR (DMSO-d_6, δ, ppm): 2.278 (s, 3H, CH_3), 3.722 (s, 3H, OCH_3), 3.794 (s, 3H, OCH_3), 4.285-4.304 (d, 2H, CH_2), 6.442-6.541 (m, 2H, CH-arom.), 7.055 (s, 1H, CH-arom.), 7.083-7.105 (t, 1H, NH), 7.437 (s, 2H, NH_2). IR (cm^{-1}): 1655 C=O, 3314 NH, 3423-3447NH_2
MO10		^{1}H NMR (DMSO-d_6, δ, ppm): 2.296 (s, 3H, CH_3), 4.452-4.471 (d, 2H, CH_2), 7.539 (s, 1H, CH-arom.), 7.489-7.518 (m, 3H, CH-arom.t), 7.563-7.601 (t, 1H, NH), 7.642 (s, 2H, NH_2). IR (cm^{-1}): 1659 C=O, 3324 NH, 3417-3438 NH_2

sitization of mice or 30 min before elicitation of the response. Only DMSO (solvent for compound) was administered to control DMSO mice. The background, non-specific inflammatory response was inducted by administration of an eliciting dose of OVA to naive mice and was subtracted from the response of sensitized mice. The results were presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice (10 measurements) and expressed in DTH units (one DTH unit = 10^{-2} cm) ± SE.

Carrageenan-induced foot pad edema

The compound **MO5** was given *i.p.* at a dose of 100 µg per mouse at 24 h and 1 h before carrageenan injection. Dexamethasone (Dex) as reference drug, was used at a dose of 40 µg, *i.p.*, at 1 h before carrageenan injection. Mice (n = 5) were given 2% carrageenan solution (100 µg in 50 µL 0.9% saline) *s.c.* into hind foot pads and after 4 h the foot pad thickness was measured by means of a spring caliper with 0.05 mm accuracy. The background foot pads thickness was induced by *s.c.* administration of 0.9% NaCl into hind foot pads of naive mice and was subtracted from the response of carrageenan injected mice. The results were presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice (10 measurements) and expressed in mm ± SE.

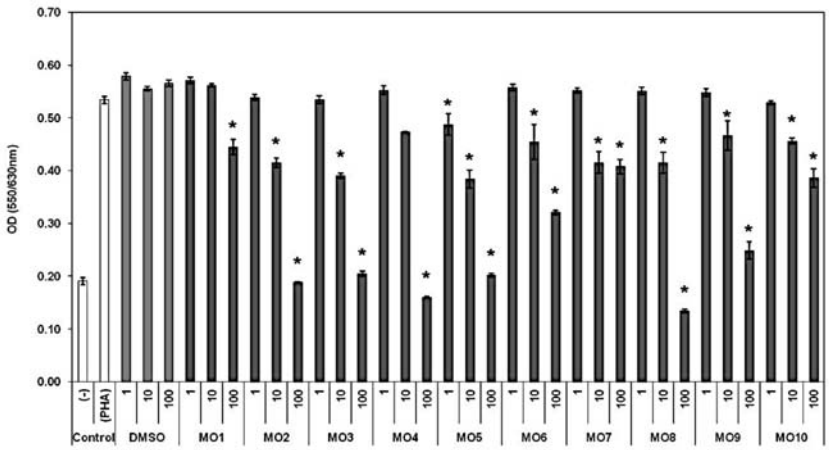


Figure 6. Effect of the compounds on PHA-induced human PBMC proliferation. The compounds were used at concentrations of 1, 10 and 100 µg/mL. The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations. The proliferative response was compared with DMSO used as solvent. *p < 0.05, when compared with DMSO cultures

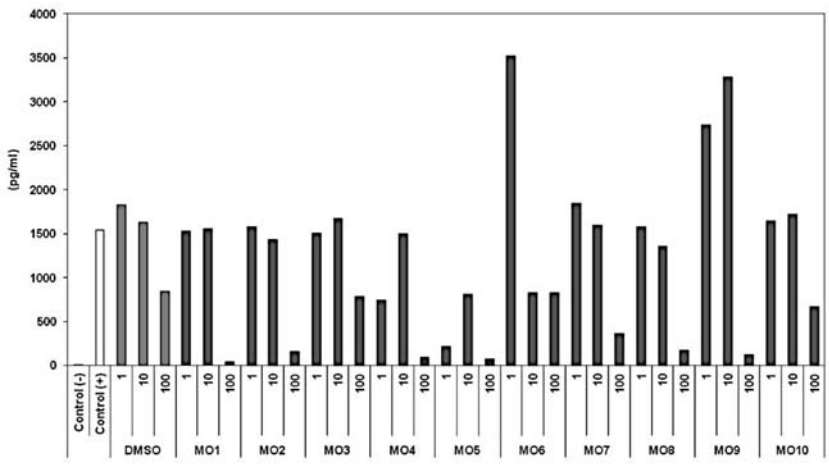


Figure 7. Effect of the compounds on LPS-induced TNF α production in human whole blood cell culture. The compounds were used at concentrations of 1, 10 and 100 µg/mL. The results are presented in pg/mL. The results were compared with DMSO used as solvent

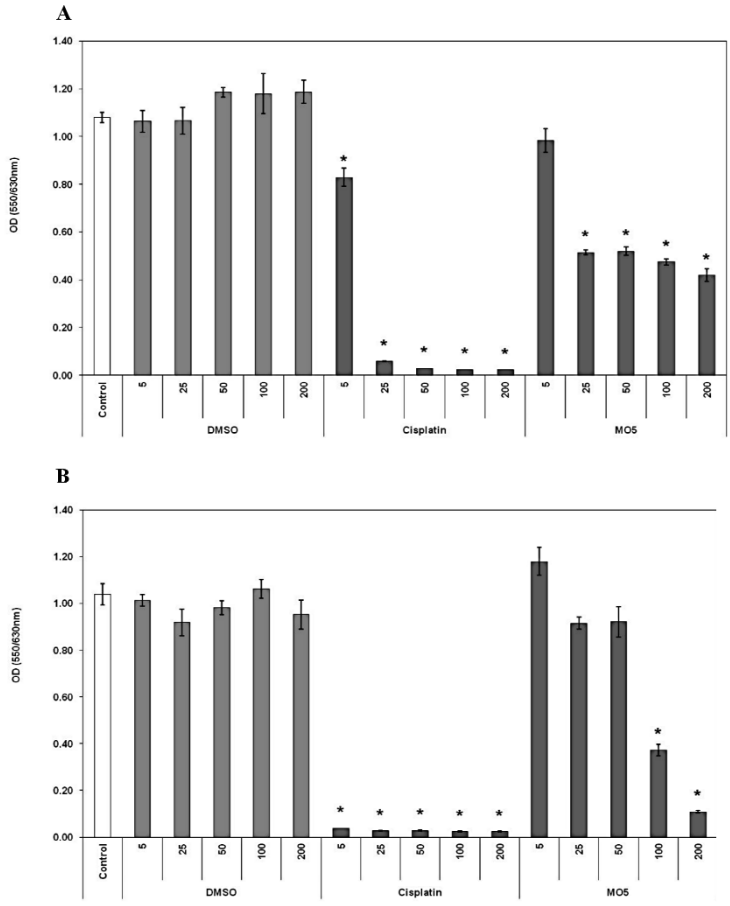


Figure 8. Effect of **MO5** on growth of SW-948 colon tumor cell (**A**) and L-1210 leukemia cell (**B**) lines. The compound **MO5** was used at concentrations of 5, 25, 50, 100 and 200 μg/mL and cisplatin was applied as reference drug at the same concentrations. The data are presented as a mean OD values at 550 nm ± SE from quadruplicate determinations. *$p < 0.05$, when compared with DMSO cultures

Data analysis and statistical procedures

The results are presented as mean values ± SE; n represents the number of mice in each of experimental group. Brown-Forsyth's test was used to determine the homogeneity of variance between groups. When the variance was homogenous, analysis of variance (one-way ANOVA) was applied, followed by *post hoc* comparisons with the Tukey's test to estimate the significance of the difference between groups. Nonparametric data were evaluated with the Kruskal-Wallis analysis of variance, as indicated in the text. Significance was determined at *$p < 0.05$. Statistical analysis was performed using STATISTICA 7 for Windows.

Chemistry

General techniques

Melting points were determined in a Büchi apparatus (Laboratoriums-Technik AG, Flawil, Switzerland), heated table Kofler system (Wagner&Munz) and were uncorrected. Thin layer chromatography (TLC) was carried out on Polygram SIL G/UV 254 nm glass silica gel plates (Macherey-Nagel), using the developing system $CHCl_3$–CH_3OH 9 : 1, v/v and detected with UV Fisher Bioblock Scientific 254 nm lamps. IR spectra were recorded with Perkin Elmer Spectrum Version 10.03.08, and ^1H NMR spectra were obtained in DMSO-d_6 using a Bruker ARX 300 MHz spectrometer (using TMS as the internal standard).

Elemental analyses were performed within ± 0.3% of the theoretical values (Carlo Erba NA, 1500 equipment).

General procedure for preparation of the compounds MO1-M10

New, not described, derivatives 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides

were synthesized from 5-amino-3-methyl-4-isoxazolecarboxylic acid azide in reaction with relevant substituted benzylamines (Scheme 1).

Five mL of propanol-2 and 5 mmol of relevant substituted benzylamines were added to 2.5 mmol of 5-amino-3-methyl-4-isoxazolecarboxylic acid azide, obtained according to a previously described method (17). The solution was stirred and heated for 4 h in temp. 82-83°C, then the reaction mixture was stirred for 24 h at room temperature. At the end of the reaction (controlled by TLC), the solid, which separated out, was filtered and washed with propanol-2. When the solid was not formed, propanol-2 was evaporated *in vacuo* from the mixture. The crude product was recrystallized from propan-2-ol. The analytical data of the obtained compounds are presented in Table 1 and the structure and spectroscopic data of the obtained compounds are presented in Table 2.

RESULTS AND DISCUSSION

In the first phase of the investigation we monitored the compounds for their possible cytotoxic effects against human PBMC. The results (Fig. 5) indicated that the studied compounds, at the concentration range of 1-100 μg/mL, did not decrease cell viability of human PBMC in 24 h cultures in comparison with respective DMSO controls. Moreover, at 100 μg/mL, the viability of cells increased. This effect was, in a way, correlated with the cell protecting effect of DMSO at 100 μg/mL.

Next, the compounds were screened for their potential inhibitory activities in the model of human PBMC stimulated in culture with PHA – the T-cell mitogen. The results shown in Figure 6 revealed differential and dose-dependent actions of the compounds on PHA-induced PBMC proliferation. The suppressive effects were already evident for the concentration of 10 μg/mL. The actions of some compounds at 100 μg/mL resulted in a total inhibition of mitogen-induced proliferation. **MO1**, **MO7** and **MO10** compounds were weakly suppressive whereas other compounds belonged to moderately acting inhibitors.

The compounds were also checked for their ability to inhibit LPS-induced TNF α production in human whole blood cell cultures (Fig. 7). The compounds displayed differential, dose-dependent, mostly suppressive (at concentration of 100 μg/mL) effects on the cytokine production. **MO6** and **MO9** compounds showed stimulatory actions at low doses. **MO5** particularly strongly suppressed TNF α production.

Taking into account relatively strong anti-proliferative properties of some compounds we wished to check effects of **MO5** on growth of two tumor cell lines (colon cancer SW-948 and lymphocytic leukemia L-1210). Cisplatin was chosen as a reference drug. The results are presented in Figure 8ab. A growth inhibitory effect (at 25 μg/mL) was relatively quickly achieved with SW-948 cell line, further increase of concentration did not, however, increase the inhibitory effect of the compound (Fig. 8a). In

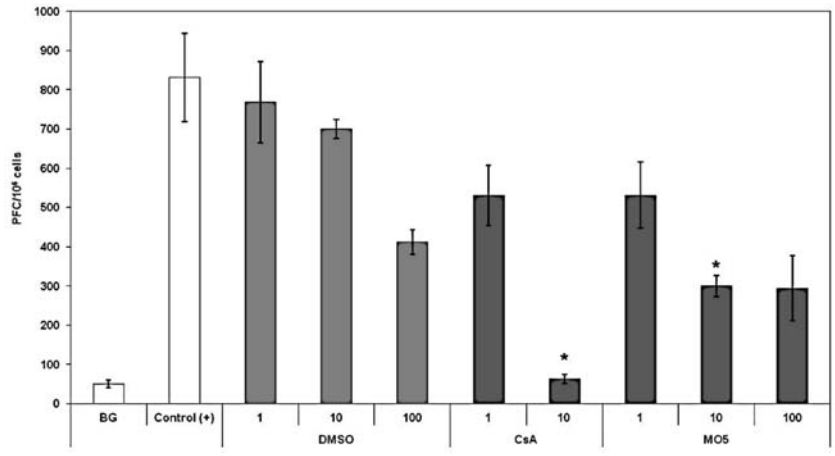


Figure 9. Effect of MO5 on the secondary humoral immune response of mouse splenocytes *in vitro* to SRBC. The results were determined using a method of local hemolysis in agar gel and are presented as the mean numbers of AFC/10^6 splenocytes ± SE. The compound MO5 was used at concentrations of 1, 10 and 100 μg/mL and CsA was applied as reference drug. *$p < 0.05$, when compared with DMSO cultures

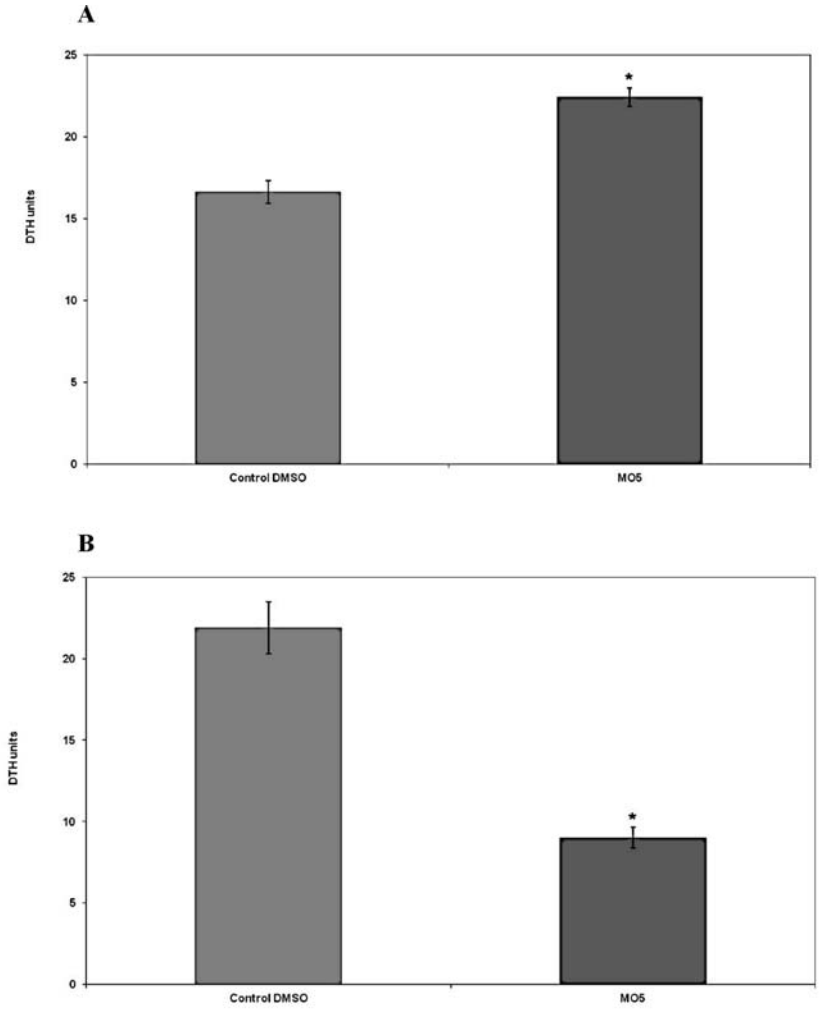


Figure 10. Effect of **MO5** on the inductive (**A**) and elicitation (**B**) phases of the DTH to OVA in mice. Mice were sensitized with OVA in cFa in tail base and 4 days later the reaction was elicited by injection of OVA in iFa in each of hind foot pads as described in Materials and Methods. The compound was administered to mice *i.p.* at 100 μg dose, 30 min before sensitization or 30 min before elicitation of the response. The results are presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice per group (10 feet/determination) and expressed in DTH units (one DTH unit = 0.01 cm) ± SE. *$p < 0.05$, when compared with DMSO control

the case of L-1210 cells (Fig. 8b) the growth suppressive effect was distinctly weaker, although dose-dependent.

Subsequently, we decided to investigate the effects of **MO5** on the antigen-specific immune response. First, the effect of **MO5** compound was checked in the model of the secondary, humoral immune response of mouse splenocytes to SRBC *in vitro*. The results presented in Figure 9 show that the suppression of the numbers of cells producing anti-SRBC antibodies by **MO5** occurred only at concentration of 10 μg/mL. No further suppression was observed at 100 μg/mL. The suppressive effect by cyclosporine A (CsA) – the reference drug – was significantly stronger (till the background levels at 10 μg/mL).

The mouse model of the delayed type hypersensitivity (DTH) *in vivo* allows to dissect effects of a given compound on induction or elicitation phases of this type of the immune response (Fig. 10ab). **MO5** was administered to mice 30 min before sensitization of animals with OVA (Fig. 10a) or 30 min before elicitation of the DTH reaction with the antigen (Fig. 10b). **MO5** enhanced the response to OVA when given before immunization (Fig. 10a). However, the compound given before the sensitizing dose of antigen strongly inhibited that type of immune response (Fig. 10b).

Lastly, we wished to investigate the effect of the compound on a nonspecific, carrageenan-induced foot pad inflammation. The mice were

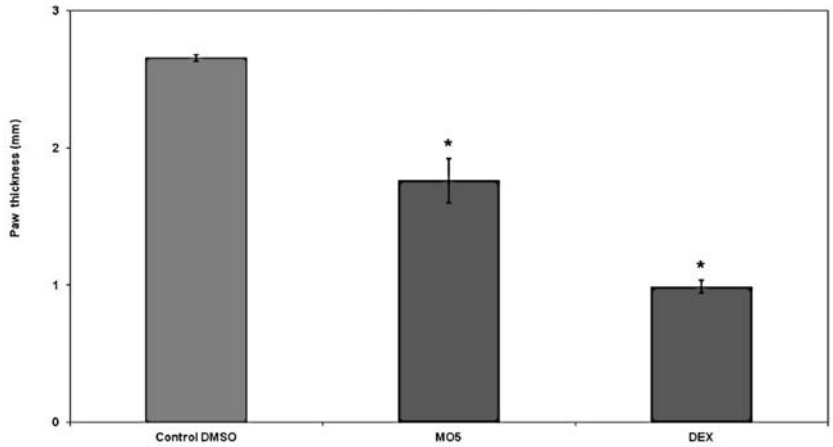


Figure 11. Suppressive effect of **MO5** on carrageenan-induced foot pad inflammation in mice. Mice were given 2% carrageenan solution *s.c.* into hind foot pads and after 4 h the foot pad thickness was measured by means of a spring caliper. The compounds were given *i.p.* at a dose of 100 μg per mouse, 24 h and 1 h before carrageenan injection. Dex was used at a dose of 40 μg, *i.p.*, at the same time. The results were presented as a mean value of antigen-specific increase of footpad thickness measured in 5 mice (10 measurements) and expressed in mm ± SE. *$p < 0.05$, when compared with DMSO control

given **MO5** compound (100 μg) *i.p.*, at 24 h and 1 h before injection of carrageenan. The compound significantly reduced the foot pad edema when measured 4 h after carrageenan administration (Fig. 11). Dexamethasone, the reference drug, given in a single 50 μg dose 1 h before carrageenan, was more potent.

DISCUSSION

In this investigation we presented anti-proliferative and anti-inflammatory properties of the studied compounds with regard to human PBMC with no apparent toxicity against these cells.

MO5, selected as most interesting compound, inhibited the secondary humoral immune response *in vitro*, suppressed the effector phase of the DTH reaction and the carrageenan reaction. Its suppressive effect may chiefly depend on inhibition of activity of inflammation mediators accompanying both antigen-specific immune response (DTH) as well as the early, nonspecific inflammatory response to carrageenan. In the latter test, **MO5** behaved similarly as RM33 (3,5,7-trimetyloizoxazolo[5,4-e]triazepin-4-on) in carrageenan-induced foot pad edema in rats (18). The inhibition of foot pad edema by **MO5** was also correlated with its ability to strongly suppress LPS-induced TNF α in the whole blood cell model. It is, therefore, likely that inhibition of pro-inflammatory cytokine production and induction of mastocyte apoptosis, as in the case of leflunomide (19), could account for a possible mechanism of action in this case. On the other hand, **MO5** stimulated the inductive phase of the cellular immune response. That interesting phenomenon could involve stimulation of antigen presentation process and/or preferential recruitment of antigen-specific T cells of Th1 type. Thus, the immunologic characteristic of the compound appears to be very intriguing. The ability to stimulate the cellular immune response by the compound administered prior to antigen may also indicate its potential adjuvant value. The structure/activity analysis of **MO5** suggests that the CH_3 group at position 4 of phenyl ring may be critical for its particularly strong activity.

At this stage of investigation, it is difficult to propose a mechanism of action for **MO** compounds. We excluded a possibility that **MO5** could be a cyclooxygenase 1 or 2 inhibitor (data not shown) as, for example another oxazolone derivative (20). Other mechanisms of its action, possibly involving arrest in a definite cell cycle, apoptosis or inhibition of NFκB expression (21-24) are likely and will be investigated in forthcoming investigations.

CONCLUSIONS

The activity screening of the series of selected 5-amino-3-methyl-4-isoxazolecarboxylic acid benzylamides and subsequent studies in the mouse

models revealed a potential therapeutic value of 5-amino-3-methyl-N-(4-methylbenzyl)-4-isoxazole-carboxamide (**MO5**). The compound exhibited strong anti-proliferative and anti-inflammatory properties. On the other hand, **MO5** had the ability to enhance manifestation of the cellular immune response. Its mechanism of action and potential therapeutic utility in more advanced experimental models remain to be established.

Acknowledgments

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NATURAL DRUGS

ASSESSMENT OF SOME BIOLOGICAL ACTIVITIES OF *ALYSSUM* L. KNOWN AS MADWORT

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Abstract: In this research the phenolic composition, and antioxidant, antibacterial and cytotoxic activities of the methanolic extracts obtained from three *Alyssum* L. taxa (*A. foliosum* var. *megalocarpum*, *A. simplex* and *A. strigosum* subsp. *strigosum*) were investigated for the first time. The antioxidant activity of the extracts was evaluated by DPPH, metal chelating, phosphomolybdenum, β-carotene/linoleic acid and ferric reducing power assays. In addition, total phenolic and flavonoid contents in the extracts were determined. The brine shrimp lethality test was used to screen for possible cytotoxic activity of the extracts. Also, the extracts were evaluated for cytotoxicity against three human cancer cell lines: MCF-7, H1299 and HeLa. HPLC analysis showed that cinnamic acid, chlorogenic acid and rutin were the major phenolics in extracts tested. Among the three *Alyssum* extracts evaluated, the highest biological activities were obtained from *A. simplex* compared to other two *Alyssum* L. taxa. Based upon its name, future studies will be targeted at investigating which components can be caused neurorehabilitation in *Alyssum*.

Keywords: *Alyssum*, biological activity, cytotoxicity, madwort, phenolic composition

Many Cruciferous species are known for their use in folk medicine for the medical care of snake bites. In addition, they are used as an antimicrobial agent for relief of wound sores and biliary colic. They have enhancing the detoxification effect of chemical carcinogen (1).

The genus *Alyssum* L. is one of the biggest genera, which has been known with up to 230 species in the world with main distribution in Turkey and Eastern Europe (2). In Turkey, this genus is represented with about 100 taxa (3). The name *Alyssum* is of Greek derivation: *a* meaning not, and *lyssa* alluding madness, rage or hydrophobia. In this direction, the names *Alyssum* and Madwort both refer to the plant's reputation as an medicinal herb. An infusion prepared from the flowers and leaves was reputed to have been administered as a specific antidote against madness or the bite of a rabid dog. Some sources have suggested the use of alyssum tea as a sedative for allaying anger (4). Shimer (5), has indicated that *Alyssum* was used by the Greeks as a remedy for hiccoughs. Various species of *Alyssum* are used in folk medicine as an expectorant, demulcent and for coughs (6, 7).

It is known that secondary metabolites are mainly responsible chemicals generated by plants that contributes to the biological potential of several plants. It is also known that the secondary metabolite compositions of plants are affected both by the variation of species and the differentiation of the environment (temperature, soil properties, UV radiation, etc.) (8). Therefore, it is expected that the three taxa of this genus contain different metabolites. The secondary metabolites of the plants such as polyphenols, flavonoids, terpenes, nitrogen-containing compounds etc. generally have antioxidant, antitumor, antimicrobial properties and they are involved in defense against reactive oxygen species (ROS), which cause a series of diseases including Alzheimer's disease, atherosclerosis, cancer and diabetes (9). The Brassicaceae is rich in a number of biologically active compounds such as glucosinolates, phenolic acids, flavonoids and vitamins, which are associated with antioxidant, antibacterial and anticancer properties (10). The glucosinolates are a large group of sulfur-containing secondary plant metabolites, which are found in the plants of the Brassicaceae family, have anti-cancer properties and block the initiation of tumors in a variety of tissues e.g., liver, bladder, pancreas, colon and small intestine. Also phenolics as antioxidants and antimicrobial compounds are well known (11).

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One of the main routes for drug development is the study of bioactivities of natural products. For this reason, this study was focused on the analysis of the phenolic content, antioxidant, antibacterial and cytotoxic activity of the methanolic extracts from three *Alyssum* taxa. The extracts were assessed for cytotoxicity against three human cancer cell lines: MCF-7 human breast carcinoma, H1299 human non-small cell lung carcinoma and HeLa human cervical carcinoma. Moreover, the extracts were screened for their possible cytotoxic activity by using brine shrimp (*Artemia salina* L.) lethality test (BSLT). Many papers have been published in which antibacterial, antioxidant and cytotoxic activities of different plant species are studied but as far as our literature survey could ascertain, there are no reports on the biological activities and phenolic composition of these *Alyssum* taxa. Thus, this study is the first record on these plants.

EXPERIMENTAL

Plant material and extraction process

Alyssum foliosum var. *megalocarpum, A. simplex* and *A. strigosum* subsp. *strigosum* were collected in the flowering stage, from Camici/Izmir-Turkey (600 m), Ahmetli/Manisa-Turkey (700 m) and Afyonkarahisar-Turkey (1020 m), respectively, and identified in our laboratory. Aerial parts of the plants were air-dried in shade at room temperature, powdered to a fine grain and then extracted with 100 mL methanol at 50°C for 6 h in a temperature controlled shaker. The extraction was repeated twice at the same conditions (12). The extracts were filtered with Whatman No. 1 filter paper and methanol was evaporated at 38°C by a rotary evaporator (IKA RV10D, Staufen, Germany). Remaining part of the extracts was dissolved with water and the water in the extract was lyophilized (Labconco FreeZone, Kansas City, MO). Resulting extracts were stored at -20°C until use.

Antioxidant activity

Total antioxidant capacity (Phosphomolybdenum method)

The total antioxidant capacity of extracts was determined by phosphomolybdenum method according to Prieto et al. (13); 0.3 mL of extract solution (1 mg/mL) was mixed with 3 mL of reagent solution (28 mM sodium phosphate, 6 M sulfuric acid and 4 mM ammonium molybdate). The reaction mixture was incubated at 95°C for 90 min. Then, the absorbance of the solution was read at 695 nm against blank. The antioxidant capacity of extract was expressed as equivalents of ascorbic acid (mgAEs/g).

DPPH free radical scavenging activity

The impact of methanol extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined according to Wu et al. (14). One milliliter of different concentrations (0.2-1.0 mg/mL) of the extracts was added to 4 mL of DPPH radical metanolic solution (0.004%). After incubation (30 min, at 25°C) in dark, the absorbances were read at 517 nm. Butylated hydroxytoluene (BHT) was used as a control. Inhibition activity was calculated in following way:

$$I(\%) = (A_0 - A_1) / A_0 \times 100$$

where A_0 is the absorbance of the control, A_1 is the absorbance of the extract/standard.

Free radical inhibition (IC_{50}) of extract was calculated. The lower the IC_{50} value indicates high antioxidant capacity.

Reducing power activity (iron (III) to iron (II) reduction)

The ferric ion reducing power assay carried out with slight modifications of the method of Oyaizu (15). Different concentrations of extracts (2.5 mL) were mixed with 2.5 mL of 1% potassium ferricyanide and 2.5 mL 0.2 M phosphate buffer (pH 6.6). The mixture was incubated for 20 min at 50°C. Then, 2.5 mL of 10% trichloroacetic acid was added and 2.5 mL of the reaction mixture was added to 0.5 mL of 0.1% ferric chloride and 2.5 mL distilled water. The solution absorbance was read at 700 nm. The results were assessed by EC_{50} values.

β-Carotene/linoleic acid assay

β-Carotene/linoleic acid assay is based on the evaluation of the inhibition of the conjugated diene hydroperoxides resulting from linoleic acid oxidation. A stock solution of β-carotene/linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL chloroform and 25 µL linoleic acid and 200 mg Tween 40 were added. Chloroform was entirely evaporated by using a rotary evaporator. Then, 100 mL of distilled water was added with vigorous shaking. Also, 2.5 mL of this reaction mixture was dispensed into test tubes and 350 µL portion (1 mg/mL) of the extract was added and the emulsion system was incubated for 2 h at 50°C. The same process was done again with synthetic antioxidant BHT, as positive control, and a blank. The absorbance of the mixtures was read at 490 nm after the incubation period and inhibition ratio was calculated (16).

Metal chelating activity on ferrous ions

The metal chelating activity on ferrous (Fe^{2+}) ions of the extracts was estimated by the method defined by Aktumsek et al. (17). Shortly, sample solution (2 mL) was added to $FeCl_2$ solution (0.05 mL, 2 mM). The reaction was started immediately by adding 5 mM of ferrozine (0.2 mL). In the same way, a blank was prepared by adding sample solution (2 mL) to $FeCl_2$ solution (0.05 mL, 2 mM) and water (0.2 mL) without ferrozine. Then, the sample and blank were left at room temperature for 10 min and the absorbances were measured at 562 nm. The metal chelating activity was expressed as equivalents of EDTA (mgEDTAs/g).

Determination of total bioactive components

Total phenolic content (Folin-Ciocalteu assay)

Total phenolic content of the extracts was analyzed *via* Folin-Ciocalteu method where gallic acid was used as a standart (18). One mL of extract solution (1 mg) was added to 46 mL of distilled water and 1 mL of Folin-Ciocalteu reagent and was mixed properly. After 3 min, the mixture was mixed with 3 mL of sodium carbonate (2%) and shaken intermittently for 2 h. The absorbance was measured at 760 nm and the total phenolic constituent was determined as equivalents of gallic acid (mgGAEs/g).

Total flavonoid content

Total flavonoid content of extracts was determined by the aluminium calorimetric method (19). Briefly, 2000 µg (1 mL of extract) was mixed with 1 mL of 2% aluminium trichloride ($AlCl_3$) in methanol. Similarly, a blank was prepared by adding extract solution (1 mL) to methanol (1 mL) without $AlCl_3$. After 10 min incubation at room temperature, the blank and extract absorbances were measured at 415 nm. The total flavonoid content was expressed as equivalents of quercetin (mgQEs/g).

Quantification of phenolic compounds by RP-HPLC

Phenolic compounds were analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). Detection and quantification were performed with a diode array detector (SPD-M20A), a LC-20AT pump, a CTO-10ASVp column heater, SIL-10ACHT auto sampler, SCL-10Avp system controller and DGU-14A degasser. Separations were carried out at 30°C on C-18 reversed-phase column (250 × 4.6 mm length, 5 µm particle size). The eluates were identified at 278 nm. The mobile phases were A: 3.0% formic acid in distilled water and B: methanol. Methanol was used to dissolve samples, and then 20 µL of this solution was injected into the column. Phenolic composition of the extract was determined according to the method of Caponio et al. (20) with slight modification. Gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, chlorogenic acid, vanillic acid, caffeic acid, *p*-coumaric acid, ferulic acid, cinnamic acid, myricetin, sinapic acid, quercetin, kaempferol and rutin (Sigma-Aldrich Co., USA) were used as standards. The differentiation and quantitative analysis were made by comparing the standards. The quantity of each phenolic compound was expressed as mg per gram of the extract.

Antibacterial assay

The extracts were tested against different microorganisms, including *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Salmonella enteritidis* (PT4), *Micrococcus luteus* (NRRLB-1013) and *Staphylococcus aureus* (ATCC 25923), by means of microdilution broth method (21). These strains were obtained from Pamukkale University Hospital. Bacterial strains were cultured on Mueller Hinton agar (MHA) at 37°C for 24 h. Test strains were suspended in Mueller Hinton broth (MHB) to give a final density of 5×10^5 cfu/mL and these were confirmed by viable counts. Dilutions ranging from 4.50 to 100 mg/mL of the extract were prepared in a 96-well microtiter plate. Plates were incubated for 24 h at 37°C. Bacterial density were then read at 610 nm to determine minimum inhibitory concentration.

Brine shrimp (*Artemia salina* L.) lethality test

Brine shrimp lethality test (BSLT) was applied to analyze the possible cytotoxic activity of the extracts. *A. salina* eggs were incubated in seawater at 28°C. After incubation for 24 h, larvae were collected and kept for an additional 24 h under the same conditions to reach the mature larvae stage. Ten larvae were drawn with a Pasteur pipette and placed in every test tube containing 4.5 mL of brine solution. In each experiment, 0.5 mL of the plant extract was mixed with 4.5 mL of brine solution and kept at 25°C for 24 h under the light and then dead nauplii were counted (22). Experiments were held together with control and five different concentrations (10, 50, 100, 500 and 1000 µg/mL) of the extract in a set of three tubes per dose. Analysis of the data was carried out by EPA Probit Analysis Program (version 1.5) to find the LC_{50} values.

Cell antiproliferation capacity

H1299 cells were cultured in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO, USA) at

37°C in a CO_2 incubator. When the cells grown to about 90% confluence the medium was discarded. Cells were washed, trypsinized, counted with a hemocytometer, and seeded into 96-well plates (1×10^3 cells/well). After 24 h incubation in the CO_2 incubator, the medium was discarded, and cells were treated with plant extract added to the medium in different concentrations (1000, 500, 250, 100, 50, 10 and 1 µg/mL) for 72 h. For the untreated control group, cells were not treated with any extracts. At the end of incubation time, medium was discarded and cytotoxicity in plant extract-treated and untreated control groups was determined by the luminometric method using a CytotoxGlo kit (Promega, Madison, WI, USA).

Statistical analysis

Experimental data were analyzed by a one way analysis of variance (ANOVA). Statistical analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL), and the significant difference between the means was compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Antioxidant potentials and phytochemical composition

Assessment of antioxidant features of plants cannot be carried out exactly by any single method because of complex nature of phytochemicals (23). Therefore, mainly five methods, including phosphomolybdenum method, β-carotene/linoleic acid bleaching method, metal chelating activity, DPPH radical scavenging activity and ferric reducing power activity were used in this study. Total phenolic and flavonoid contents of the extracts were also evaluated as gallic acid and quercetin equivalents, respectively (Fig. 1), because the phenolic compounds such as flavonoids and phenolic acids are known as strong chain breaking antioxidants and may contribute directly to antioxidative activity (24). The results of the antioxidant activities of three *Alyssum* taxa are summarized in Table 1.

Free radical scavenging activity represented as IC_{50} values and BHT was the reagent used as standard in the assay. IC_{50} value is the effective concen-

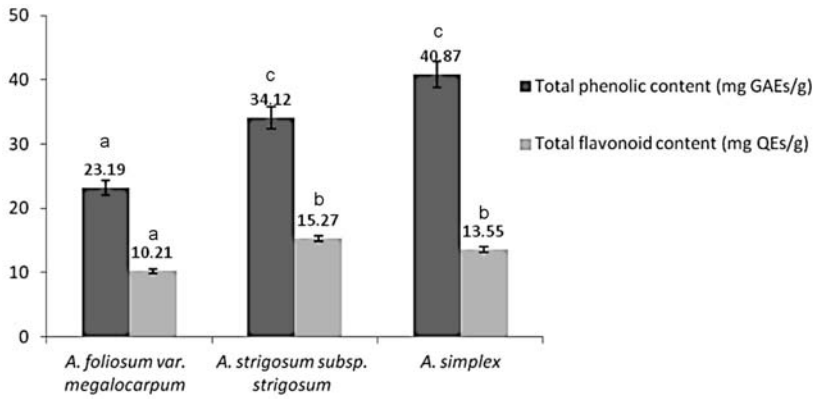

Figure 1. Total phenolic and flavonoid contents in three *Alyssum* taxa (mean ± SD). Different letters indicate significant difference ($p < 0.05$). (GAEs = gallic acid equivalents, QEs = quercetin equivalents)

Table 1. Antioxidant activities of three *Alyssum* L. taxa (mean ± SD).

Plants	β-Carotene / linoleic acid assay (%)	DPPH assay (IC_{50} mg/mL)	Ferric reducing power activity (EC_{50} mg/mL)	Phosphomolybdenum assay (mgAEs/g)[a]	Metal chelating activity (mg EDTAEs/g)
A. strigosum subsp. *strigosum*	73.45 ± 1.25[a]	0.117 ± 0.006[c]	0.143 ± 0.002[c]	68.13 ± 1.76[a]	11.04 ± 0.05[a]
A. foliosum var. *megalocarpum*	67.11 ± 1.12[a]	0.123 ± 0.004[c]	0.149 ± 0.005[c]	71.81 ± 1.15[a]	10.01 ± 0.07[a]
A. simplex	83.58 ± 0.07[b]	0.102 ± 0.001[b]	0.135 ± 0.003[b]	80.21 ± 1.25[b]	17.11 ± 0.13[b]
BHT	94.03 ± 0.67[c]	0.018 ± 0.002[a]	0.061 ± 0.001[a]	nt	nt

[a]AEs = ascorbic acid equivalents; [b]EDTAEs = EDTA equivalents, nt = not tested; different letters in the same column indicate significant difference ($p < 0.05$).

Table 2. Phenolic components in the methanolic extracts from three *Alyssum* L. taxa (mg/g extract) (mean ± SD).

No.	Phenolic components	*A. simplex*	*A. strigosum* subsp. *strigosum*	*A. foliosum* var. *megalocarpum*
1	Gallic acid	0.42 ± 0.02^{b}	0.39 ± 0.03^{b}	0.40 ± 0.03^{b}
2	Protocatechuic acid	0.09 ± 0.01^{a}	0.11 ± 0.01^{a}	0.05 ± 0.02^{a}
3	4-Hydroxybenzoic acid	0.31 ± 0.02^{b}	0.25 ± 0.02^{b}	0.32 ± 0.02^{b}
4	Chlorogenic acid	0.51 ± 0.01^{c}	0.48 ± 0.04^{b}	0.97 ± 0.05^{c}
5	Vanillic acid	0.37 ± 0.03^{b}	0.27 ± 0.02^{b}	0.09 ± 0.01^{a}
6	Caffeic acid	0.35 ± 0.02^{b}	1.32 ± 0.04^{c}	0.87 ± 0.03^{c}
7	*p*-Coumaric acid	0.28 ± 0.01^{b}	0.03 ± 0.01^{a}	0.17 ± 0.01^{a}
8	Ferulic acid	0.21 ± 0.01^{a}	0.11 ± 0.01^{a}	0.06 ± 0.01^{a}
9	Cinnamic acid	1.05 ± 0.04^{c}	0.29 ± 0.02^{b}	0.02 ± 0.01^{a}
10	Rutin	1.15 ± 0.05^{c}	0.86 ± 0.03^{c}	0.75 ± 0.04^{b}
11	Myricetin	0.08 ± 0.01^{a}	nd	nd
12	Sinapic acid	0.14 ± 0.02^{a}	nd	0.10 ± 0.01^{a}
13	Quercetin	0.17 ± 0.01^{a}	0.12 ± 0.03^{a}	0.11 ± 0.02^{a}
14	Kaempferol	0.90 ± 0.02^{c}	0.87 ± 0.02^{c}	0.77 ± 0.03^{b}

Different superscript letters in the same row indicate significant difference ($p < 0.05$); nd = not determined.

tration at which DPPH radicals were scavenged by 50% and was estimated from the graph plotting extract concentration against inhibition percentage. IC_{50} value is inversely related to antioxidant capacity of the extracts. In the present study, *A. simplex* extract showed greater antioxidant activity than that of *A. foliosum* var. *megalocarpum* and *A. strigosum* subsp. *strigosum* extract with an IC_{50} value of 0.102 mg/mL (Table 1). Fe^{3+} reduction is often used to determine electron donation activity which is an important mechanism of antioxidants. Therefore, in order to evaluate the electron-donating powers of *Alyssum* extracts, their ability to reduce Fe (III) was studied. Reducing power activity was assessed by using EC_{50} (the effective concentration at which the absorbance was 0.5). Lower EC_{50} value indicates higher antioxidant capacities. Reducing power of tested *Alyssum* extracts seemed to be correlated in a similar manner with free radical scavenging activity (Table 1). There were no significant differences in ferric reducing activities between *A. foliosum* var. *megalocarpum* and *A. strigosum* subsp. *strigosum* extracts ($p > 0.05$) but *A. simplex* extract exhibited significant difference ($p < 0.05$).

Total antioxidant activities of tested *Alyssum* extracts were examined by β-carotene/linoleic acid and phosphomolybdenum assays. Phosphomolybdenum method is based on the reduction of Mo (IV) to Mo (V) by the antioxidants and the subsequent formation of green phosphate/Mo (V) compounds with a maximum absorption at 695 nm. In this assay, the highest activity was demonstrated by *A. simplex* extract (80.21 mgAEs/g) followed by *A. foliosum* var. *megalocarpum* (71.81 mgAEs/g) and *A. strigosum* subsp. *strigosum* (68.13 mgAEs/g) extract (Table 1). The ability of the *Alyssum* extracts to inhibit lipid peroxidation was investigated using the β-carotene/linoleic acid bleaching assay. From the point of inhibition potential of linoleic acid oxidation, three *Alyssum* taxa can be ranked from high to low in the following order: BHT (94.03%) > *A. simplex* (83.58%) > *A. strigosum* subsp. *strigosum* (73.45%) > *A. foliosum* var. *megalocarpum* (67.11%). However, inhibition values of *A. strigosum* subsp. *strigosum* and *A. foliosum* var. *megalocarpum* were not statistically different ($p > 0.05$). It is most likely that the antioxidant components such as phenolics in *A. simplex* extract can decrease the grade of β-carotene destruction by inhibiting the linoleic acid oxidation in the test system.

Metal ion chelating activity of each plant was tested against ferrous ion. The chelating activities of the extracts were established using EDTA as a standard (mg EDTAEs/g extract). In good agreement with results of other antioxidant assays, potent chelation capacities were again detected in the *A. simplex* extract with 17.11 mg EDTAEs/g (Table 1) ($p < 0.05$). Since ferrous ions were the most effective pro-oxidant for lipid peroxidation, *A. simplex* extract would be useful. Within this scope, the

capacity of substances or plant extracts to chelate iron can be important mechanism for antioxidant activity (25).

Among phytochemicals possessing antioxidant activity, phenolic compounds are one of the most important groups (26). The highest phenolic content was determined in *A. simplex* extract (40.87 mgGAEs/g). In the same way, total flavonoid contents were found to be high in *A. simplex* and *A. strigosum* subsp. *strigosum* extracts (Fig. 1). The results of total phenolic content demonstrated a uniform tendency to those of the antioxidant capacities. Accordingly, the high content of total phenolics in the extract might explain the strong antioxidant capacity of *A. simplex*. These results are in good agreement with previously published reports in the literature, which exhibited strong connection between antioxidant activities and total phenolic content (9, 27). Similar results were reported for some *Alyssum* species such as *Alyssum homolocarpum* by Souri et al. (7). In a previous study, where 35 selected plants from Spain were compared in terms of their antioxidant activity, total phenolic content and skin care properties, *A. serpyllifolium* subsp. *lusitanicum* leaf extract has exhibited high antioxidant activity and phenolic contents (28). The key role of phenolic compounds as scavengers of free radicals is accentuated in several reports (29).

As a part of the study, a rapid high performance liquid chromatographic method using a reversed-phase column and a diode array detector (HPLC-DAD) method was established. Using HPLC analysis, the content of the major phenolic compounds of three *Alyssum* L. taxa extracts are listed in Table 2. Main phenolic compounds were determined as cinnamic acid (0.02-1.05 mg/g extract), chlorogenic acid (0.48-0.97 mg/g extract) and rutin (0.75-1.15 mg/g extract). These data indicated that the biological activities of three *Alyssum* L. taxa could be attributed to their polyphenol compounds. Rutin is well known as antioxidant and as natural compound with large spectrum of medicinal properties (30). Therefore, high concentration of rutin was thought to be responsible for the biological activities found. In addition, chlorogenic acid content was closely correlated to biological activities such as inhibition of lipid peroxidation and metal chelating (31).

Antibacterial activity

The antibacterial activities of three *Alyssum* taxa were analyzed by microdilution broth method. The obtained results are shown in Table 3. The minimum inhibitory concentration (MIC) values of the extracts were found at 12.5-100 mg/mL concentration range. *A. simplex* extract was found as the most effective extract against *P.aeruginosa* at a concentration of 12.5 mg/mL. This is followed by *A. strigosum* subsp. *strigosum* and *A. foliosum* var. *megalocarpum* (25 mg/mL). Generally, the cell walls of Gram-negative bacteria, which are more complex than Gram-positive ones, act as a diffusional barrier and make them less susceptible to the antibacterial agents than the Gram-positive bacteria. Despite this permeability difference, methanolic extracts of *Alyssum* taxa exerted some degrees of inhibition against Gram-negative bacteria as well. It can be concluded that polar protic solvents may be useful

Table 3. MIC values (mg/mL) of *Alyssum* extracts against standard bacteria.

Microorganisms	*A. simplex*	*A. strigosum* subsp. *strigosum*	*A. foliosum* var. *megalocarpum*	Gentamicin	Oxacillin
Escherichia coli (ATCC 25922)	-	-	50	3.12	-
Pseudomonas aeruginosa (ATCC 27853)	12.5	25	25	3.12	-
Salmonella enteritidis (PT4)	-	25	-	1.56	-
Staphylococcus aureus (ATCC 25923)	-	-	-	-	6.25
Bacillus subtilis (NRRL B-209)	100	-	-	-	3.12
Micrococcus luteus (NRRLB-1013)	50	50	25	-	6.25

Table 4. Human cancer cell lines and BSLT results for three *Alyssum* L. taxa.

Plants	Human cancer cell lines[a]			
	MCF-7	H1299	HeLa	BSLT[b]
A. strigosum subsp. *strigosum*	277.02	264.09	125.18	37.50
A. foliosum var. *megalocarpum*	262.11	250.32	119.35	139.72
A. simplex	216.15	235.08	63.05	29.22

[a] Cytotoxicity results are expressed as IC_{50} values (µg/mL; concentration at which 50% inhibition occurs). [b] BSLT results are expressed as LC_{50} values (µg/mL; concentration at which 50% lethality occurs).

for increasing antibacterial activity of Gram negative bacteria. There are several chemical components present in plants with antimicrobial effect including saponin, phenolics, flavonoids, glucosinolates and organic acids. However, the major components in plants with antimicrobial activity are phenolic compounds such as aliphatic alcohols, aldehydes, ketones and isoflavonoids (32). For instance, the antibacterial activity of 46 extracts from medicinal herbs and spices was suggested to be associated with the presence of phenolic constituents (33). A previous study reported that methanolic extracts of *Alyssum* spp. exhibited significant antioxidant and antibacterial activity (34). Our antimicrobial results were also in agreement with the literatures that showed a good correlation between antimicrobial and antioxidant activity (35). On the other hand, a study has revealed that *Alyssum maritimum* leaf extracts act as a natural quorum sensing inhibitor with slight inhibition (36).

Cytotoxic activity

The results of cytotoxic activity of three *Alyssum* L. taxa extracts using BSLT are shown in Table 4. The extract of *A. simplex* exhibited significant cytotoxicity with LC_{50} value of 29.22 µg/mL, which indicates it more toxic than the other two *Alyssum* extracts. The mortality for each extract increased with increasing the concentration. In a previous study, the LC_{50} value of *Lepidium sativum* L. (Brassicaceae) extract was determined as 262.46 µg/mL (37). The screenings for possible cytotoxic activity of plant extracts using BSLT were reported previously (38, 39). We concluded that the brine shrimp (*A. salina*) lethality test has been recommended as an acceptable method to screen the cytotoxic activity of plant extracts.

Cancer is one of the most important human diseases, and there is considerable scientific and commercial interest in the discovery of new anticancer agents from natural sources (40). There are many difficulties associated with its treatment, the most common of which include drug resistance, toxicity and the low specificity of currently available cytotoxic drugs (41). These difficulties promote the importance of research aimed at the identification and development of novel anticancer compounds. The effect of the crude methanolic extracts of three *Alyssum* L. taxa on the growth of three human cancer cell lines was determined by the luminometric method using a CytotoxGlo kit. We found that *A. simplex* has cytotoxic effects on MCF-7, HeLa and H1299 cell lines and IC_{50} values are 216.15, 63.05 and 235.08 µg/mL, respectively (Table 4). These results are consistent with BSLT results. The BSLT represents a rapid, cheap and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity and anti-tumor properties (22).

CONCLUSIONS

In conclusion, such detailed studies on biological activity and chemical composition of three *Alyssum* taxa have been performed for the first time. Thus, their chemical composition was determined indicating significant amounts of phenolics, rutin in particular. In terms of the biological activity assays, antioxidant, antibacterial, and cytotoxic activity of the extracts were revealed. *A. simplex* extract possesses more remarkable biological properties than the other two *Alyssum* taxa. A high positive relationship was observed between biological activities and phytochemical composition. Referring to the *Alyssum*'s common name, future studies will be aimed at examining which components in *Alyssum* can cause neurorehabilitation.

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ANTIOXIDANT, ANTIBACTERIAL AND GUT MODULATING ACTIVITIES OF *KALANCHOE LACINIATA*

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Anstract: *Kalanchoe laciniata* (L.) DC. (Crassulaceae) is a widely distributed plant in Africa and Asia. Traditionally, various communities use this plant for the treatment of a variety of ailments such as gut problems, allergic conditions and wounds. The current study was designed to explore the antibacterial, antioxidant and gut modulating activities of *K. laciniata* in order to provide scientific rationale for its traditional uses. Phytochemical compounds were assessed through screening 70% crude methanolic extract of *K. laciniata*. Its gut modulatory activity was evaluated by *in vitro* tissue experiments on rabbit jejunum which yielded maximal spasmogenic response of 28.4 ± 4.6% (n = 4) at 3 mg/mL, while spasmolytic response was recorded with EC_{50} value of 3.2 mg/mL (2.8-3.5, 95% CI, n = 5). In antibacterial assays crude extract was found effective against *Staphylococcus aureus* and *Bacillus subtilis,* with MIC value of 5 and 2.5 mg/mL, respectively. The testing of the methanolic crude extract for antioxidants resulted in total phenolic contents of 27.8 ± 1.8 mg GAE/g DW and 22.7 ± 2.1 mg AAE/g DW total antioxidant activity. It also scavenged 17.3 ± 3.0% of DPPH free radical when compared with quercitin.

Keywords: spasmolytic, spasmogenic, calcium channel blocking, cholinergic

Kalanchoe laciniata (L.) DC. (local name: Zakhm-e-Hayat) is an erect branched herb of family Crassulaceae, which is predominantly found in Africa and tropical Asia. The leaves of *K. laciniata* are widely used in Asia for counter-irritant remedies because of their astringent and antiseptic properties (1). In India, Philippines, Cambodia, Vietnam and Malaysia its leaves are used topically on wounds, to relief headache and cough, treat ulcer and to reduce body temperature. The juice of its leaves is taken orally in case of diarrhea, while the whole plant can be taken as a tea to reduce heart discomfort and gastric pain (2-4). Traditional healers suggest this plant in skin allergy, bronchitis, asthma and impaired digestion. Southeastern Ethiopians use the root extract of fresh plant intranasally as a remedy for tonsillitis (5).

A plenty of pharmacological attributes have been described in different species of genus *Kalanchoe*, such as *Kalanchoe pinnata* which possesses antitumor, antimicrobial, antiviral, antiulcer, anti-inflammatory, antileishmanial, immunosuppressive, antioxidant and hepatoprotective activities (6). *Kalanchoe crenata* have analgesic, anticonvulsant (7), and antihyperglycemic activities (8), while *Kalanchoe brasiliensis* showed antithyroid and immunosuppressive activities (9, 10). This clearly indicates that the genus *Kalanchoe* is quite rich in medicinal properties. Despite the wide range of reported long-established uses of *K. laciniata*, there is no evidence of any pharmacological research over this plant. Hence, the study was aimed to investigate antimicrobial, antioxidant and calcium channel blocking activities of crude extract of *K. laciniata* to provide scientific justification for its conventional uses.

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MATERIALS AND METHODS

Plant material and extract preparation

The dried aerial parts of *K. laciniata* were purchased from the herbal market of Lahore. Its identity was confirmed by the botanist Mr. Abdul Hameed at Cholistan Institute of Desert Studies, the Islamia University of Bahawalpur, and a specimen having voucher no: KL-AP-07-10-008, was submitted at the herbarium of Faculty of Pharmacy and Alternative Medicine, the Islamia University of Bahawalpur. About 500 g of plant material was cleaned from dirt, coarsely crushed and then it was immersed three times in 70% methanol and filtered initially by muslin cloth followed by Whatman No. 1 filter paper. All the filtrates were accumulated together and evaporated under reduced pressure at 35-40°C. After removal of solvent, solidified sticky brownish crude extract of *K. laciniata* (Kl.Cr) was retrieved with a yield of about 17.51% and was stored at -20°C.

Chemicals and reagents

All the chemicals used in this study were of analytical grade. NaCl, $CaCl_2$, KCl, $MgCl_2$, NaH_2PO_4, $NaHCO_3$, Na_2CO_3, Mueller Hinton agar and nutrient broth were purchased from Merck Millipore Darmstadt, Germany. Na_3PO_4, $(NH_4)_2MoO_4$, H_2SO_4, glucose, EDTA, 2,2-diphenyl-1-picrylhydrazyl (DPPH), verapamil, gallic acid, ascorbic acid, acetylcholine, Folin-Ciocalteu reagent and quercetin, were purchased from Sigma-Aldrich Co., while ampicillin and gentamicin antibiotic discs were purchased from Thermo Fisher Scientific Inc.

Animals and bacterial strains

Rabbits weighing 1-1.5 kg were purchased from local breeders, while albino mice having weight of 20-25 g, were obtained from National Institute of Health, Pakistan. The animals were kept at animal house under hygienic environment and provided with food and water. The experiments were performed in accordance with the guide for the care and use of laboratory animals (11). *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, kindly provided by the Department of Biochemistry and Biotechnology, the Islamia University of Bahawalpur, were used to evaluate the antimicrobial activity of plant extract.

Phytochemical screening

Phytochemical contents of Kl.Cr were explored to detect the presence of saponins, alkaloids, flavonoids, tannins, anthraquinones, glycosides, steroids, ketones, monosaccharaides, carbohydrates and soluble starch by using standard methods as previously described by our research group (12).

In vitro isolated tissue experiments

In vitro tissue experiments were performed as described previously (13). On the day of experiment, a 24 h fasting rabbit was operated, its jejunum was removed and placed in pre warmed Tyrode solution (37°C), aerated with carbogen gas (5% CO_2 and 95% O_2). The jejunum tissue was cut into 1.5-2 cm pieces, cleaned from fatty tissues and fixed in 50 mL organ bath, filled with Tyrode solution (37°C) and continually aerated with carbogen gas. The jejunum tissue was equilibrated for 30 min and then treated with sub maximal strength of acetylcholine (0.3 µM) to stabilize the contractions. After stabilization, the effect of different concentrations of Kl.Cr on tissue was recorded by using student kymograph (MTA-786/1024). Spasmogenic effect was quantified by comparing with maximum contraction induced by high dose of acetylcholine (1 µM), while the spasmolytic response of Kl.Cr was observed by calculating the percentage reduction in spontaneous contractions. Spasmolytic response was further investigated by depolarizing jejunal tissue with high potassium (80 mM). After achieving sustained contractions, the tissue was treated with Kl.Cr in a cumulative manner to record relaxant effect on smooth muscles and percentage relaxation was calculated with reference to maximum contraction by high potassium. It is established fact that substances which inhibit high potassium induced contractions are also calcium influx blockers (14). To confirm that calcium channel blockade is the underlying mechanism for smooth muscle relaxing effect of Kl.Cr, we stabilized rabbit jejunum tissue in normal Tyrode solution, and then tissue was decalcified by incubating for 30 min in calcium-free Tyrode solution containing 0.1 mM EDTA. After 30 min, solution was changed with calcium-free, potassium-rich Tyrode solution and again allowed to stay for 30 min. Then, controlled $CaCl_2$-concentration curves were constructed and re-constructed until two superimposable curves were produced. The tissue was treated for 1 h with Kl.Cr, and again $CaCl_2$-induced curve was recorded. This whole procedure was performed with different concentrations of Kl.Cr and also with verapamil as a standard drug.

Agar disc diffusion assay

Antimicrobial effect of Kl.Cr was determined by the same method as previously described but

with some modifications (15). For agar disc diffusion assay, bacteria were cultured overnight in a nutrient broth at 37°C, then diluted to 1×10^8 colony forming units with sterile nutrient broth. This diluted bacterial culture was evenly spread on freshly prepared and solidified Mueller Hinton agar plates with the help of sterile cotton swab. By using sterile forceps, 6 mm filter paper discs each containing 10 mg of Kl.Cr, standard antibiotic discs of ampicillin (10 µg) and gentamicin (10 µg) were placed on agar plates at appropriate distance and incubated for 18-24 h at 37°C. On the next day, inhibition zones were measured and this experiment was repeated twice.

MIC and MBC determination

The minimum inhibitory concentration (MIC) was determined for bacterial strains which were sensitive to Kl.Cr in agar disc diffusion assay, by using previously described method (16). Briefly, 12 h old bacterial culture was adjusted with nutrient broth at 0.5 McFarland standard. Kl.Cr was solubilized in sterile water (20 mg/mL), followed by two fold serial dilutions ranging from 10 to 0.3 mg/mL. Then, 95 µL of nutrient broth and 5 µL of pre-diluted bacterial culture were added in 96 well plates under sterile conditions. A 100 µL of Kl.Cr initially prepared at concentration of 20 mg/mL was added into first well followed by 100 µL of each of serial dilutions added to the next wells so that final volume in each well became 200 µL. Ampicillin/gentamicin were used as standards, while nutrient broth and pure bacterial culture served as negative and positive control, respectively. For each bacteria separate plate was used to avoid the cross contamination. The plates were covered with lid, shook for 5 min on shaker, and then incubated for 24 h at 37°C. On the next day, plates were observed and confirmation of growth inhibition was done by sub-culturing 5 µL from clear wells, on nutrient agar for 24 h at 37°C. Experiments were performed three times and MIC was described as minimum concentration of Kl.Cr which caused inhibition of bacterial growth. Minimum bactericidal concentration was the lowest concentration which exhibited no growth after this re-plating.

Estimation of total phenolic contents

Total phenolic contents of Kl.Cr were estimated by the same procedure as described earlier (17). Pre-diluted Kl.Cr (125 µL) was mixed with Folin-Ciocalteu reagent (125 µL) and water (500 µL). Then, 1250 µL of Na_2CO_3 (7%) was added and the mixture was shaken. Final volume was made up to 3 mL with distilled water, and incubated in dark at 23°C for 90 min. After incubation, absorbance was measured at 760 nm, and phenolic contents were determined through calibration curve of gallic acid (0-500 µg/mL). The experiment was repeated three times.

Estimation of total antioxidant capacity

Phosphomolybdenum method was used to determine the total antioxidant activity of Kl.Cr as described (18). Briefly, 3 mL of reagent solution (28 mM sodium phosphate, 4 mM ammonium molybdate and 0.6 M sulfuric acid) was mixed with 0.3 mL of properly diluted Kl.Cr and incubated for 90 min at 95°C. After cooling it down to room temperature, absorbance was measured at 695 nm, while ascorbic acid served as standard. The experiment was performed three times.

DPPH radical scavenging assay

Kl.Cr was evaluated for its radical scavenging ability by using previously described method (19), with minor modifications. Ninety µL of DPPH solution (100 µM) was mixed with 10 µL of pre-diluted crude extract in 96-well plate and incubated for 30 min, at room temperature in dark place. After incubation, absorbance was measured at 517 nm, while quercitin served as standard antioxidant. The experiment was performed in triplicate. Percentage radical scavenging was calculated by applying the following formula:

Scavenging activity (%) = 100 – [Absorbance of test compound/Absorbance of control] ×100

Acute toxicity testing

To determine the toxicity of Kl.Cr we followed the preceding method (13). Mice were randomly divided into four groups and each group contained five animals. Group 1, 2, and 3 were orally given increasing concentrations of Kl.Cr; i.e., 0.3, 1 and 3 g/kg, respectively, while group 4 was given normal saline (0.9% NaCl, 10 mL/kg). The animals were observed for 24 h and allowed to have food and water *ad libitum*.

Statistical analysis

Statistical analysis was done by using Graph PAD prism 5.01 software. Results were presented as the mean ± standard error of the mean (SEM), and EC_{50} at 95% confidence interval (CI), with p value equal or lower than 0.05, while "n" shows number of experiments performed.

RESULTS

Phytochemical tests performed on Kl.Cr indicated that it contains flavonoids, saponins, glyco-

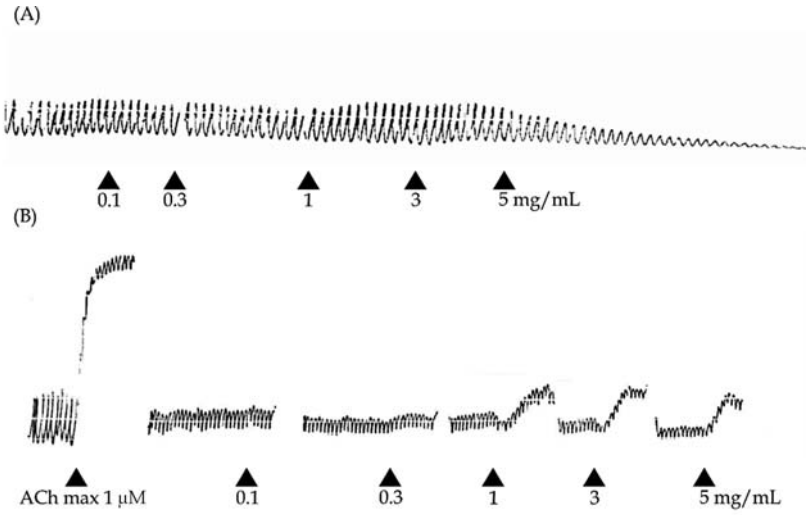

Figure 1. (A) Spontaneous contractions and spasmolytic activity of *K. laciniata* crude extract checked in the presense of atropine. (B) spasmogenic response of this extract checked in the absence of atropine

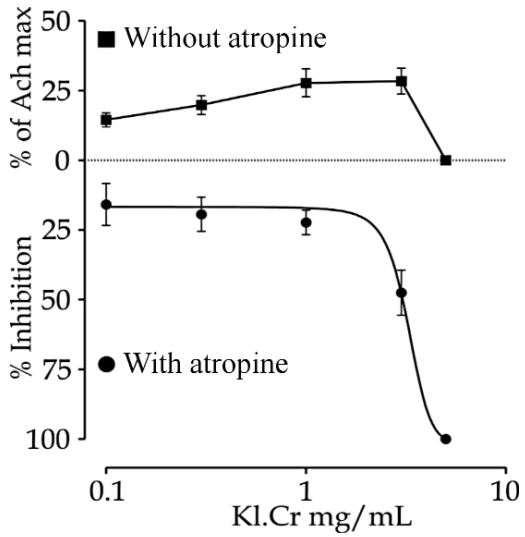

Figure 2. Spasmogenic and spasmolytic effect of *K. laciniata* crude extract on isolated rabbit jejunum tissue with (n = 5) and without atropine (n = 4)

sides, tannins, carbohydrates, steroids, soluble starch and pentoses. The crude extract of *K. laciniata* exhibited both spasmogenic and spasmolytic response when it was checked on spontaneously contracting rabbit jejunum tissue (Fig. 1). At concentrations of 1, 3 and 5 mg/mL, initially spasmogenic response was observed, which was followed by spasmolytic activity, the maximum contraction was induced at 1 and 3 mg/mL of Kl.Cr. The contractile response was assessed by comparing it with maximal dose of acetylcholine (1 µM), which was 27.7 ± 4.9% and 28.4 ± 4.6% (n = 4) of acetylcholine induced maximum contraction for 1 and 3 mg/mL, respectively (Fig. 2). To investigate the underlying mechanism of spasmogenic response we atropinized the jejunal tissue with 1 µM atropine. In atropinized tissue, spasmogenic response was abolished and Kl.Cr exhibited spasmolytic activity only, having EC_{50} value of 3.2 mg/mL (2.8-3.5, 95% CI, n = 5) as shown in Figure 2. The possible mechanism behind the spasmolytic effect of Kl.Cr was investigated by treating the jejunal tissue with high potassium (80 mM) to generate the sustained contractions, and then Kl.Cr was added in cumulative manner. The sustained contraction was completely relaxed at concentration of 5 mg/mL with EC_{50} value of 3 mg/mL (2.6-3.4, 95% CI, n = 4). It has been already established that calcium channel blockers can relax potassium induced contractions. So, spasmolytic activity was further investigated by constructing calcium-response curves in calcium-free and potassium-rich Tyrode solution. Kl.Cr shifted the calcium-response curve towards right at concentrations of 0.3 and 1 mg/mL (n = 4). This shift in curve was similar when compared with verapamil at concentration of 0.03 and 0.1 µM (n = 4) as shown in Figure 3, which confirmed calcium channel blocking activity of Kl.Cr.

In antibacterial activity, growth of *S. aureus* and *B. subtilis* was inhibited by Kl.Cr, but it was not effective against *E. coli*. The growth inhibitory effect was more pronounced on *B. subtilis* as compared to that of *S. aureus*. In agar disc diffusion assay, the zone of inhibition for *B. subtilis* was 14.6

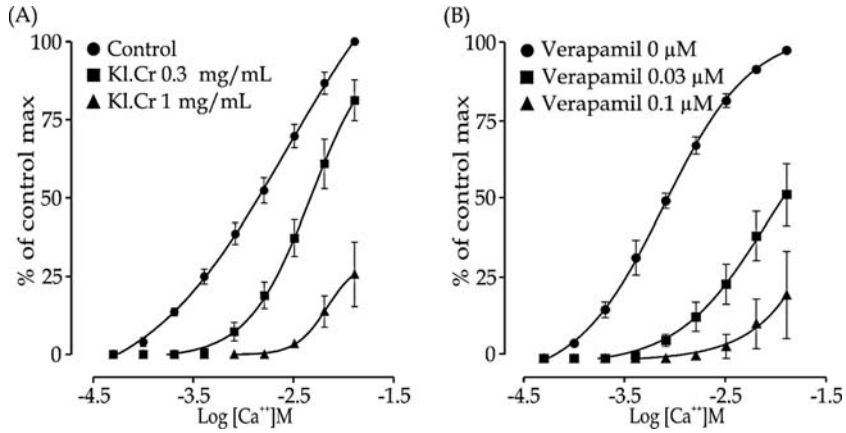

Figure 3. (A) Calcium-response curves constructed on isolated rabbit jejunum tissue in the presence and absence of different concentrations of *K. laciniata* crude extract. (B) Calcium-response curves in isolated rabbit jejunum tissue with and without verapamil. Values are shown as the mean ± SEM of 4 experiments

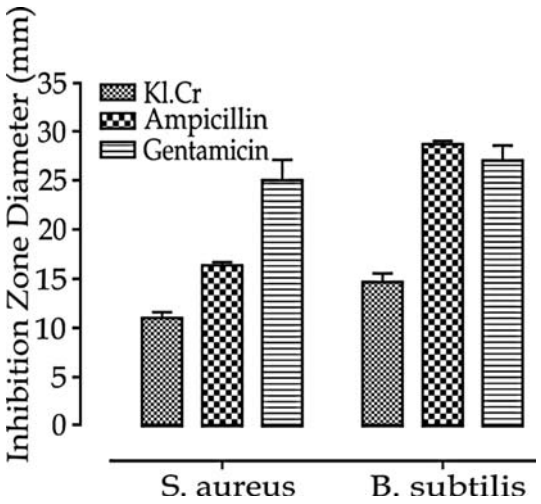

Figure 4. Comparison of inhibition zone diameter against *S. aureus* and *B. subtilis* by the *K. laciniata* crude extract, ampicillin and gentamicin. Values are shown as the mean ± SEM of three replicates

± 1.5 mm and for *S. aureus* it was 11 ± 1 mm (Fig. 4). Minimum inhibitory concentration of Kl.Cr was also determined, which was 5 and 2.5 mg/mL for *S. aureus* and *B. subtilis*, while 10 and 5 mg/mL were minimum bactericidal concentrations for *S. aureus* and *B. subtilis*, respectively.

In antioxidant assays, total phenolic contents of Kl.Cr were found 27.8 ± 1.8 mg equivalents of gallic acid per gram of crude plant extract while total antioxidant activity of Kl.Cr was 22.7 ± 2.1 mg ascorbic acid equivalents per gram of dry extract, which is comparable with total phenolic contents. So, antioxidant activity of Kl.Cr might be considered due to its phenolic contents. Finally, the radical scavenging potential of Kl.Cr was 17.3 ± 3.0% and 95.6 ± 0.01% for 0.1 mg/mL of Kl.Cr and quercitin, respectively (Fig. 5). Kl.Cr did not exhibit any lethal effect on mice in acute toxicity testing, so it was considered safe till the dose of 3 g/kg *p.o.*

DISCUSSION

Gut motility is the coordinated movement of gastrointestinal smooth muscles. This coordinated movement is responsible for normal processing of food we eat, while any abnormality in gastrointestinal movements results in disorders such as spasm, constipation and diarrhea. *K. laciniata* has been traditionally known for its use in treating dysentery, diarrhea and dyspepsia, so it was evaluated to provide pharmacological basis for its conventional uses. *In vitro* tissue experiments of Kl.Cr performed on isolated rabbit jejunum exhibited both spasmogenic and spasmolytic response. Spasmogenic action of crude plant extracts can be of cholinergic or non-cholinergic type, which can be differentiated by testing the plant extract in the presence of a relatively high concentration of the anti-cholinergic drug (20). The spasmogenic effect of Kl.Cr was completely abolished by the presence of atropine indicating the cholinergic mechanism. It has already been described that gastrointestinal smooth muscle contractions are mediated by M_3 receptors which are main muscarinic receptors in GIT (21), so it might be possible that Kl.Cr induces contractile response by interacting with M_3 receptors. Spasmolytic activity of crude plant extract is usually mediated by blockade of calcium channels (22, 23). Calcium channel blockers constitute an important

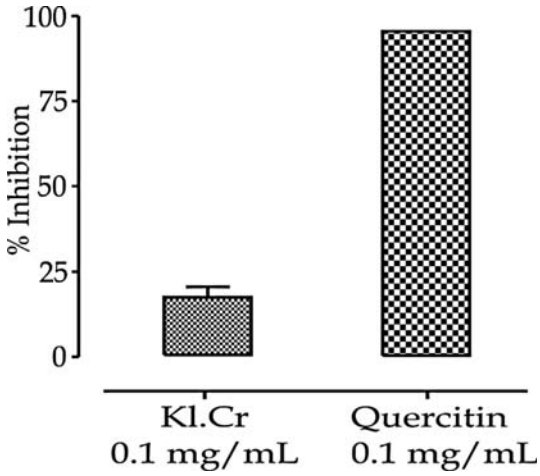

Figure 5. DPPH free radical scavenging activity of *K. laciniata* crude extract and quercetin. Values are shown as the mean ± SEM of three replicates

therapeutic group, they characteristically inhibit the slow entry of calcium in a dose dependent manner, which is reversed by calcium (24). Calcium-response curve constructed with Kl.Cr was almost identical to that of verapamil, which confirmed that Kl.Cr possesses calcium channel blocking activity. Both the cholinergic and calcium channel blocking activities of Kl.Cr can be considered due to the presence of saponins and flavonoids as these substances are reported to exhibit cholinergic and calcium channel blocking activities (13, 25).

Kl.Cr contained flavonoids, saponins, and tannins as indicated by its phytochemical analysis. Flavonoids and related polyphenols play an important role in protecting plants against microbial invasion (26), and nowadays polyphenols are gaining much focus due to their wide biological activities. Several recent studies have reported that flavonoids and flavonones isolated from plants were found to possess antimicrobial activity (27, 28) and the possible mechanism is thought to be metal ion deprivation, interaction with enzymes and destruction of microbial membranes (29). Tannins also exhibit toxicity to microorganisms by complexing various enzymes such as cellulases, pectinases, xylanases, peroxidases, laccase, and glycosyl transferase (30, 31), while Gram negative bacteria were found to be less sensitive than Gram positive bacteria, due to the coating of lipopolysaccharides on the surface of Gram negative bacteria which may repulse the phenolic compounds (32). Saponins also take part in plant defense systems and it was described before that they possess antimicrobial activity (33). Their mechanism of antibacterial action is not well understood yet, but it is known that Gram positive bacteria are more susceptible to saponins as compared to Gram negative bacteria, which is attributed to glucosidase enzyme synthesized in Gram negative bacteria that may degrade the saponins (34). In view of above discussion, it can be proposed that Kl.Cr exerts antimicrobial activity due to the presence of tannins, flavonoids and saponins and it is more active against Gram positive bacteria. Tannins and flavonoids also possess antioxidant activity and metal chelating properties. They are used therapeutically for their antioxidant, anti-inflammatory, wound healing, and anti-microbial activities (35).

CONCLUSION

It is concluded from the experimental data that crude extract of *K. laciniata* contains both concentration dependent cholinergic and calcium channel blocking activity, which accounts for its spasmogenic and spasmolytic response. It also possesses antibacterial activity against common skin pathogen *S. aureus* and antioxidant activity which provides reason for its traditional use in wound healing and skin problems.

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Declaration of interest

The authors declare no conflict of interest.

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EFFECT OF JASMONIC ACID ON TOTAL PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF EXTRACT FROM THE GREEN AND PURPLE LANDRACES OF SWEET BASIL

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Abstract: Basil (*Ocimum basilicum* L.) belongs to the family Lamiaceae and is an important aromatic and medicinal plant, which it widely cultivated in many countries. This plant is a good source of phenolic compounds and natural antioxidants. The main aim of present study was to determine effect of jasmonic acid on total phenolic content and antioxidant activity of ethanolic extract from the aerial parts of the purple and green landraces of basil. The pot experiment was conducted in an experimental field with cold and semiarid climate in southwestern Iran. Treatments comprised control (water), ethanol as solvent, 200 and 400 µL jasmonic acid. The total phenolic content of the extract by Folin-Ciocalteu method and the antioxidant activity using DPPH assay were determined. Results indicated that the different levels of jasmonic acid had significant effects on total phenolics content and antioxidant activity of the extracts. Foliar-applied jasmonic acid in particular 400 µL increased total phenolic content in the plants as compared to untreated plants. In conclusion, it is suggested that jasmonic acid as an abiotic elicitor could be a promising material used to increase biological activity and pro-health functional value of basil plants.

Keywords: *Ocimum basilicum* L., ethanolic extract, elicitor, jasmonic acid, antioxidant activity, total phenol

Among various medicinal herbs, some species are of particular interest because they may be used for the production of materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits (1). The basil herb (*Ocimum basilicum* L.) is one of the most frequently used pharmacological materials. These days, basil is cultivated worldwide, in particular because it is a rich source of natural compounds, such as monoterpenes, sesquiterpenes, phenylpropanoids, anthocyanins, and phenolic acids (2). Extract and essential oil from the aerial parts of basil have been used extensively in the food industry as a flavoring agent, and in perfumery and medical industries (3-5). Basil has also shown antialergic, anticancer, antimicrobial, antiseptic, antispasmodic, antifungal, antiviral, anti-inflammatory, analgesic, immune-stimulating, sedative and antioxidant activities due to its polyphenols and aromatic compounds (6-10).

Phenolics in vegetables and herbs are the major bioactive compounds known for health benefits especially due to their antioxidant properties. The antioxidant activity of phenolic compounds is mainly caused by their redox properties, which permit them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (11). The main phenolics reported in basil are phenolic acids (e.g., rosmarinic, lithospermic, vanillic, p-coumaric, hydroxybenzoic, syringic, ferulic, protocatechuic, caffeic and gentisic), and flavonol-glycosides (12). Therefore, basil extracts are used in the cosmetic and pharmaceutical products.

Recently, scientists have done extensive research to increasing polyphenols concentration in plants to further enhance their overall nutritional and pharmaceutical value (6). Elicitation is a technique that stimulates plants to accumulate secondary metabolites. It has been reported that various elicitors interact with plant membrane receptors and generate signal compounds that induce expression of genes encoding enzymes of secondary metabolites biosynthesis and can also have an indirect effect on

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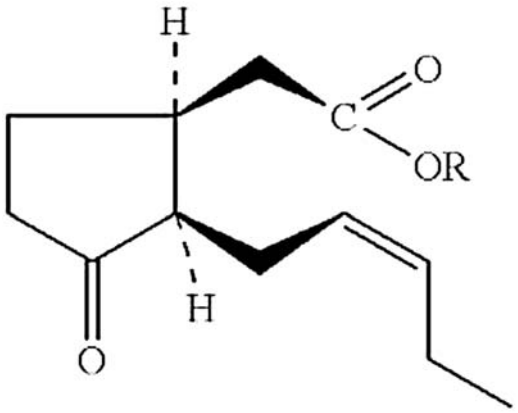

Figure 1. Chemical structure of jasmonic acid (R = H)

phenolics accumulation in plants (13). For instant, total phenolic content in romaine lettuce (*Lactuca sativa* L.) increased after methyl jasmonate treatments (14). In addition, methyl jasmonate treatment dramatically increased the levels of some secondary metabolites in *Nicotiana attenuata* leaves (15). Jasmonic acid (Fig. 1), is a potent elicitor or signaling agent and plays key roles in plant growth and affecting a great diversity of physiological and biochemical processes (16). In this study, the effect of abiotic elicitor jasmonic acid on the content of phenolics and antioxidant properties of green and purple basil was investigated.

EXPERIMENTAL

Chemicals and reagents

Gallic acid, Na_2CO_3, methanol and ethanol used in this study were purchased from Merck Co. (Darmstadt, Germany). The Folin-Ciocalteu reagent, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (Steinheim, Germany).

Plant materials and growth conditions

The seeds of the green and purple landraces of sweet basil were purchased from Pakan Seed Company. Pot experiments were conducted at the Research Field, Islamic Azad University of Shahrekord (latitude. 32° 20´ N; longitude 50° 51´ E; altitude 2070 m asl.), southwestern Iran, until May to August 2014. The seeds of two Iranian landraces of basil were sown in plastic pots with a diameter of 20 cm and a depth of 25 cm. The climate of the area study was cold, semiarid and semi humid with temperate summer by Emberger's climatology method and very cold winter by Karimi's climatology method (17). The pots were filled with clay loam with a pH of 7.23, containing 0.8% organic matter comprised of 0.01% total N, 11.20 mg/kg available phosphorus, 694 mg/kg available potassium, and a saline value measured at E.C.: 1.35 dS/m. The plants were sprayed with water (control), ethanol as solvent, and different levels of jasmonic acid (200, and 400 µL) thrice at 10-12 leaves, before flowering, and two weeks later. These solutions were sprayed at dew point (approximately 100 mL per plant) with a hand sprayer (untreated control plants were sprayed with an equivalent volume of distilled water) for two Iranian landraces, including green and purple basil.

Extract preparation

The aerial parts of the plants were shade dried and ground into a powder (50 g), macerated in 100 mL of ethanol 70% and filtered and then were dried at 35°C under rotary vacuum (Model Zirbus 302w, Italy). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use.

Determination of total phenolic content (TPC)

The total amount of phenolic compounds in each extract was determined using the Folin-Ciocalteu method following procedure of Singleton and Rossi (18) with some modifications. Briefly, 0.5 mL of the sample were mixed with 2.5 mL of Folin-Ciocalteu's phenol reagent and kept for 5 min at 37°C. Then, 2 mL of saturated Na_2CO_3 (7.5%) was added, and the mixture was brought to 10 mL with the addition of deionized, distilled water. The mixture was maintained at room temperature in the dark for 120 min and then the absorbance of the mixture was measured at 765 nm against a reagent blank using a UV–Vis spectrophotometer (Perkin-Elmer Lambda, US). Gallic acid equivalent (GAE) was used as the reference standard and results were expressed as milligrams of gallic acid equivalents per gram of each extract on dry basis (mg GAE/g).

Antioxidant test

The antioxidant capacity of the extracts was evaluated by the method of Brand-Williams et al. (19). The extracts at different concentrations (16 to 500 µg/mL) were mixed with the same volume of 0.2 mM methanol solution of DPPH. The disappearance of DPPH by extracts after 30 min of incubation at room temperature was determined spectrophotometrically at 515 nm. Methanol was used to zero spectrophotometer. The absorbance of the DPPH radical without antioxidant, i.e., the control was measured

daily using a Perkin–Elmer Lambda UV/VIS spectrophotometer at 515 nm against a blank, i.e., without DPPH. All tests were run in triplicate and an average was used. Decreasing of DPPH solution absorbance indicates an increase of DPPH radical scavenging activity. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC_{50}) was calculated graphically and the percentage inhibition was calculated according to the equation:

$$\% \text{ inhibition} = [\frac{A_{C(0)} - A_{A(t)}}{A_{C(0)}}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 30 min.

Statistical analysis

Simple and interaction effects of experimental factors were derived from two-way analysis of variance (ANOVA) based on the GLM procedure of the SAS statistical package (SAS/STAT® v.9.2. SAS Institute Inc., Cary, NC). The assumptions of variance analysis were tested by ensuring that the residuals were random and homogenous, with a normal distribution about a zero mean. The significance of differences among treatment means was tested using Duncan's multiple range test (DMRT) at $p \leq 0.05$.

RESULTS AND DISCUSSION

Total phenolic content

The conducted studies aimed at determining the influence of jasmonic acid on the content of phenolic compound in the green and purple landraces of basil. Results of statistical analysis showed significant differences in the total phenolic content of the extracts of basil under treatment of jasmonic

Table 1. Effect of foliar application of jasmonic acid and landraces on total phenolic content and antioxidant activities of basil.

Treatments	**TPC** (mg GAE /g extract)	**IC_{50}** (µg/mL)
	Foliar application	
Water (Control)	0.1931d	1544.0a
Solvent (ethanol)	0.1941c	1539.0b
Jasmonic acid (200 µL)	0.1974b	1509.1c
Jasmonic acid (400 µL)	0.1986a	1501.2d
ANOVA	$p \leq 0.01$	$p \leq 0.01$
Landraces		
S1(Green)	0.1896b	1534.7a
S2 (Purple)	0.2021a	1511.9b
ANOVA	$p \leq 0.01$	$p \leq 0.01$

Letters correspond with bars in Figure 1.

Table 2. The interaction effect of jasmonic acid × landrace on total phenolic content and antioxidant activities of basil.

Treatments	**TPC** (mg GAE /g extract)	**IC_{50}** (µg/mL)
J1 × S1	0.1879g	1505.7e
J1 × S2	0.2003c	1572.3a
J2 × S1	0.1868h	1521.9c
J2 × S2	0.1994d	1566.0b
J3 × S1	0.1934e	1504.0e
J3 × S2	0.2014b	1514.1d
J4 × S1	0.1902f	1515.9d
J4 × S2	0.2071a	1486.6f
ANOVA	$p \leq 0.01$	$p \leq 0.01$

Letters correspond with bars in Figure 2.

acid. Table 1 shows variations in the phenolic compound existing among the different jasmonic acid treatments of basil. The content of total phenolics from the two landrace of basil were significantly increased after the application of different levels of jasmonic acid (Tables 1, 2). Stimulation of total phenolic compounds in plant tissues after jasmonic acid treatment was found earlier (16, 17). This result is also similar with Kim et al. (22) study where at the second day after the treatment by 0.1 and 0.5 M methyl jasmonate total phenolic contents were 27 and 57%, respectively, higher than in the control. Similarly, Cai et al. (23) have reported that treatment with elicitores efficiently enhanced biosynthesis of phenolic compounds in *Vitis vinifera*.

Synthesis of phenolic compounds is recognized initiated very quickly after elicitation. Overall, elicitors such as jasmonic acid are considered to be plant signaling molecules that are involved in some signal transduction systems, and induce gene expression levels of enzymes of the secondary metabolic pathway such as PAL (phenylalanine

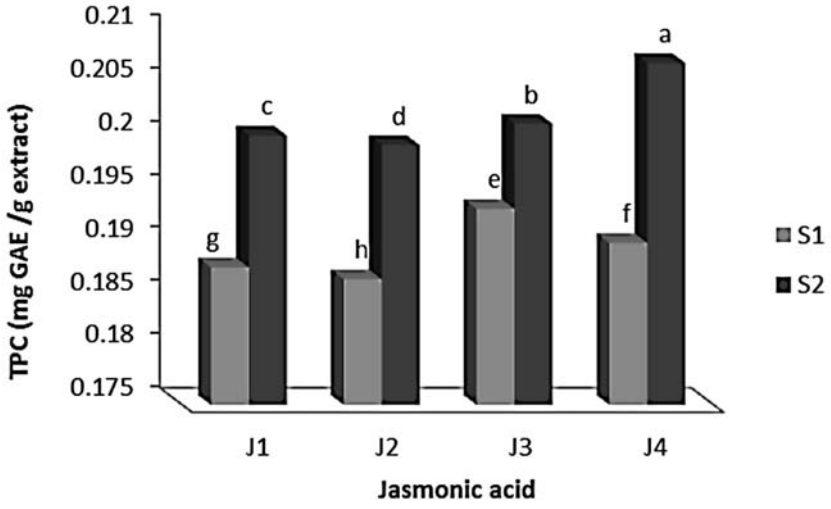

Figure 2. Effect of jasmonic acid on total phenol content in green and purple basil

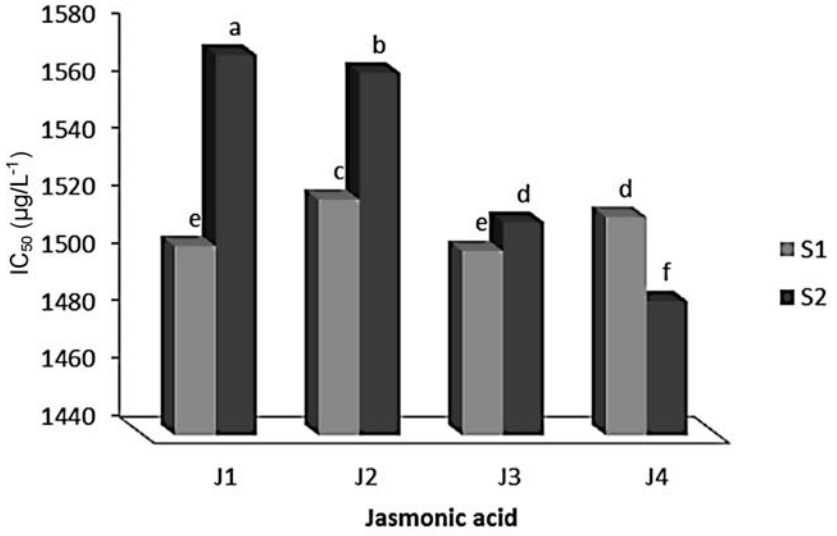

Figure 3. Effect of jasmonic acid on IC_{50} (µg/mL) in the green and purple landraces of basil

ammonia lyase), an enzyme involved in the synthesis of phenolics through phenylpropanoid pathway and in consequence increase the amount of phenolic compounds (24-26). The initial step of phenylpropanoid synthesis is mediated by phenylalanine ammonia lyase (PAL) enzyme. An increase in PAL activity could often be considered as a marker of plant reaction to elicitors. In addition, jasmonate induces other of genes involved in biosynthesis of phenolics such as polyphenol oxidase (27).

Plants phenolics present in herbs, because of their potential antioxidant, antimutagenic and antitumor activities, have received considerable attention (28). Phenolic compounds, due to their antioxidant activities and free radical scavenging abilities, are widely distributed in plants (29), which have gained much attention and potentially have beneficial implications for human health (30). Therefore, phenolic compounds are the major group contributing to the antioxidant activity of vegetables, fruit, cereals and other plant-based materials. The antioxidant activity of phenolics is mainly due to their redox properties, which make them acting as reducing agents, hydrogen donors, and singlet oxygen quenchers (31).

In this experimental work, the interaction effect of jasmonic acid × landraces, also had a significant effect on total phenolic content. The maximum total phenolic content was obtained by foliar spray of jasmonic acid (400 µL) for purple basil (Table 2).

Antioxidant activity

The antioxidant activity of the green and purple basil extracts was determined by measuring their ability to remove free DPPH radicals present in a methanol solution. The DPPH is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants (32). The lower IC_{50} value indicates a stronger ability of the extract to act as a DPPH scavenger, while the higher IC_{50} value indicates a lower scavenging activity of the scavengers as more scavengers are required to achieve 50% scavenging reaction. Results of this study showed that the extract from purple basil had higher antioxidant activity than green basil, which have maximum amount of phenolic compounds (Table 1).

Our results are in accordance with a previous report, which has shown that higher phenolic compound levels increase the antioxidant activity (33) and also showed a linear correlation between phenolics contents and antioxidant activity (34). There is a positive correlation between phenolics content and antioxidant properties in plants (35). The antioxidant activity in basil is attributed both to its extract and soluble phenolic fractions. The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play an important role in neutralizing ROS, such as free radicals, singlet and triplet oxygen and peroxides (36). Because of the high relative antioxidant activity of basil, this plant can be a good source for pharmaceutical industries and new sources of antioxidant phenolics in the diet.

Results of this study also indicated that spray jasmonic acid treatments had a significant impact ($p \leq 0.01$) on the antioxidant activity (Table 1). The most antioxidant activity was exhibited by the extract from the plants under the foliar spray of 400 µL jasmonic acid (Table 1). Our results correspond with the study of Kim et al. (37) that indicated methyl jasmonate treatment resulting in the increased antioxidant capacities determined by DPPH assay in lettuce leaves. Moreover, in agreement with our results, Bandurska et al. (38) reported exogenous application of jasmonic acid or methyl jasmonate to increase antioxidative ability of plants. Treatment with signalling molecules like jasmonic acid may induce H_2O_2 production, which in turn, may induce the synthesis or activation of various transcription factors and are associated with the induction of different antioxidant enzymes (39). Overall, jasmonic acid could regulate the activities of antioxidant enzymes (40) and can affect the antioxidant system in plant cells (41).

CONCLUSIONS

In conclusion, this study has demonstrated that amounts of phenolic compounds and antioxidant activity of extracts of green and purple basil increased by the jasmonic acid treatment as compared to those in the control. These results emphasized the importance of abiotic elicitors for enhancement of the phenolic compounds and the antioxidant activity of basil plant extracts, which might be alternative and effective means instead of genetic modification. Overall, with regard to the results of this study, the extract of green and purple basil could be an important source of phenolic compounds with antioxidant capacity, nonetheless, in order to gain better views on the antioxidant levels in basil, further studies on purification, identification and quantification of each phenolic compound and other non-phenolic compounds are necessary in the future.

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ANTI-OXIDATIVE, α-GLUCOSIDASE AND α-AMYLASE INHIBITORY ACTIVITY OF *VITEX DONIANA*: POSSIBLE EXPLOITATION IN THE MANAGEMENT OF TYPE 2 DIABETES

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Abstract: *Vitex doniana* is an important African medicinal plant traditionally used for the treatment of many diseases including type 2 diabetes (T2D). In this study, ethyl acetate, ethanol and aqueous extracts of the stem bark, root and leaf of *V. doniana* were analyzed for *in vitro* anti-oxidative activity and the results indicated that the ethanolic extract of the leaves had the best anti-oxidative activity. Subsequently, the ethanolic extract of the leaves was partitioned between hexane, dichloromethane, ethyl acetate and water. The aqueous fraction had a significantly ($p < 0.05$) higher phenolics content and also showed the best anti-oxidative activity within the fractions. Furthermore, the aqueous fraction demonstrated significantly ($p < 0.05$) more potent inhibitory activities against α-glucosidase and α-amylase than other fractions. Steady state kinetics analysis revealed that the aqueous fraction inhibited both α-glucosidase and α-amylase activities in a non-competitive manner with inhibition binding constant (Ki) values of 5.93 and 167.44 µg/mL, respectively. Analysis of the aqueous fraction by GC-MS showed the presence of resorcinol, 4-hydroxybenzoic acid, 3,4,5-trimethoxyphenol and 2,4'-dihydroxychalcone identified by their mass fragmentation patterns and comparison to standard spectra. The results obtained in this study showed that *V. doniana* leaves have a good *in vitro* anti-T2D potential possibly elicited through phenolics.

Keywords: anti-oxidative activity, α-glucosidase, α-amylase, *Vitex doniana*, type 2 diabetes

Despite extensive research efforts, the incidence of diabetes is still increasing at an alarming rate with over 346 million people affected worldwide and the number is expected to rise to 544 million people in 2030 (1, 2). Prolonged diabetes leads to serious damage to many of the body's physiological processes and causes a number of medical complications, such as cardiovascular disease, stroke, atherosclerosis, blindness, kidney damage, lower limb amputations among many others (2, 3). Among the two major types of diabetes, type 2 diabetes (T2D) accounts for 90-95% of the total diabetic patients worldwide. It is a heterogeneous disorder characterized by a progressive decline in insulin action (insulin resistance), followed by the inability of pancreatic β-cells to compensate for insulin resistance (β-cell dysfunction) which leads to hyperglycemia (4).

Prolonged hyperglycemia leads to the auto-oxidation of glucose and formation of advanced glycated end products which are involved in the generation of reactive oxygen species (ROS) that cause lipid peroxidation and play an important role in the production of secondary complications in T2D (5). Oxidative stress is believed to be a common pathway linking diverse mechanisms for the pathogenesis of microvascular and macrovascular complications of diabetes (6). Therefore, the use of antioxidants could be exploited in the treatment of T2D and prevention of its chronic vascular complications (7). Another therapeutic approach commonly exploited in the management of T2D is decreasing the post-prandial rise of blood glucose level by impeding glucose absorption in the digestive tract through inhibition of carbohydrate hydrolyzing enzymes (α-glucosidase and α-amylase) (8). Combined actions of α-glucosidase and α-amylase enzymes are responsible for hydrolysis of starch, complex carbohydrates and oligosaccharides to glucose and other monosaccharides which are then absorbed in the

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intestinal epithelium and enter into the blood circulation (9). Therefore, α-glucosidase and α-amylase inhibitors will ultimately reduce the flow of glucose from complex dietary carbohydrates into the bloodstream, diminishing the postprandial hyperglycemia.

At present, many clinical drugs are used as α-glucosidase inhibitors; however, severe side effects such as diarrhea, flatulance, lactic acid intoxication and other gastrointestinal problems are associated with their intake (10, 11). Randomized controlled trials with glucosidase inhibitors report these gastrointestinal side effects as the most common reason for noncompliance and early subject withdrawal (12). Interestingly, plant based agents were reported to be a more acceptable source of glucosidase inhibitors due to their low cost and non-toxic nature, including a low incidence of serious gastrointestinal side effects (13, 14). On the other hand, synthetic anti-oxidative agents, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propylgallate (PG), and tert-butylhydroxytoluene exhibit potent free radical scavenging effects but they induce liver damage and carcinogenesis in laboratory animals (15, 16). Based on the above, recourse to plants as sources of antioxidants and α-glucosidase inhibitors becomes an appealing alternative.

Vitex doniana (Verbanaceae) commonly called African black plum is widely distributed in tropical West Africa. Various parts of the plant are used by traditional medicine practitioners in Nigeria for the management and treatment of several disorders which include rheumatism, hypertension, cancer, and inflammatory diseases. An ethnobotanical study also revealed that the plant leaves are used in the treatment of diabetes mellitus in northern Nigeria (17). The myelo- and hepatoprotective as well as the analgesic and antitrypanosomal activities of the leaf extracts have also been reported in several recent studies (18-21). The stem bark of the plant was found to induce graded uterine muscle contractions (22) and demonstrated antidiarrhoeal and antibacterial activities (23-25). Furthermore, natural consumption of the plant was linked to reduced reproductive function of wild baboons (26). A recent preliminary study also reported the antioxidant potential of the methanolic extract from the leaves of this plant (27). However, the detailed anti-oxidative effects of different parts of this plant using various extracts and solvent fractions as well as the inhibitory effects on the carbohydrate digesting enzymes are still unknown, despite the traditional use of the plant in the management of diabetes.

Hence, our current study conducted a comprehensive and systematic investigation on the *in vitro* anti-oxidative as well as α-glucosidase and α-amylase inhibitory activities of various extracts and solvent fractions of *V. doniana* with a view to find new alternative agent(s) that could provide a holistic avenue to control postprandial hyperglycemia and other diabetic complications.

MATERIALS AND METHODS

Chemicals and reagents

Yeast α-glucosidase, porcine pancreatic amylase, p-nitrophenyl-α-D-glucopyranoside (pNPG), p-nitrophenol, acarbose, gallic acid, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 2-deoxy-D-ribose, ascorbic acid and potassium ferricyanide were obtained from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Starch, dinitrosalicylic acid (DNS), maltose, absolute ethanol, ethyl acetate, trichloroacetic acid, hydrogen peroxide, ferric chloride, Griess reagent, sodium nitroprusside, thiobarbituric acid and Folin-Ciocalteau reagent were obtained from Merck Chemical Company, Durban, South Africa.

Plant material

The stem bark, root and leaf samples of *Vitex doniana* were freshly collected in the month of January, 2011 from Zaria, Kaduna state, Nigeria. The plant was authenticated at the herbarium unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria and a voucher specimen number 1162 was deposited accordingly. The stem bark, root and leaves were immediately washed and shade-dried for about two weeks to constant weights. The dried samples were pounded to fine powder using a kitchen blender, and then stored individually in air-tight containers for transport to the University of KwaZulu-Natal, Durban South Africa for subsequent analysis.

Preparation of crude extracts and solvent partitioning of crude ethanolic extract of the leaves

Forty (40) grams of the fine powdered sample from each part of the plant were separately defatted with hexane. The defatted materials were sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 h in 200 mL of the respective solvent followed by shaking for 2 h at 200 rpm. Then, after filtration through Whatmann filter paper (No. 1), the respective solvents were evaporated under vacuum using a rotary evaporator (Buchi Rotavapor II,

Buchi, Germany) at 40°C under reduced pressure to obtain the solvent extracts. Aqueous extracts were dried on a water bath at 50°C. Based on the results of the preliminary anti-oxidative assays (described below), the most active ethanolic extract of the leaves was further prepared *via* fractionation with solvents of different polarity. A 10 g of the crude ethanolic extract of the leaves was dissolved in a 200 mL of distilled water : methanol (9 : 1, v/v) and successively partitioned with hexane (2 × 200 mL), dichloromethane (2 × 200 mL), ethyl acetate (2 × 200 mL) and n-butanol. However, the n-butanol completely dissolved the remaining aqueous fraction after ethyl acetate partitioning and therefore the fraction was regarded as aqueous fraction. The resulting fractions were evaporated to dryness at 40°C under reduced pressure, whereas the aqueous fraction was dried on a water bath. The crude extracts and the fractions in each case were weighed, transferred to micro tubes and stored at 4°C until further analysis.

Estimation of total phenolics content

The total phenolics content of each extract or fraction was determined according to the method described by McDonald et al. (28) with slight modifications. Briefly, 200 µL of the extract or fraction (240 µg/mL) was incubated with 1 mL of Folin-Ciocalteau reagent (diluted 10 times) and 800 µL of 0.7 M Na_2CO_3 for 30 min at room temperature. Then, the absorbance values were determined at 765 nm on a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate and the total phenolics content in each extract or fraction was calculated as gallic acid equivalent.

Ferric cyanide (Fe^{3+}) reducing antioxidant power (FRAP) assay

The FRAP method of Oyaizu (29) was used with slight modifications to measure the reducing capacity of the extracts and fractions. To perform this assay, 1 mL of each extract or fraction (15–240 µg/mL) was incubated with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50°C for 30 min. After incubation, the reaction mixture was acidified with 1 mL of 10% trichloroacetic acid. Thereafter, 1 mL of the acidified solution was mixed with 1 mL of distilled water and 200 µL of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. An increased absorbance of the reaction mixture indicates a greater reduction capability of the extracts. The results were expressed as a percentage of the absorbance of the sample to the absorbance of gallic acid:

$$\text{Ferric reducing antioxidant power (\%)} = \text{Absorbance of sample/Absorbance of gallic acid} \times 100$$

Free radical scavenging activity (DPPH method)

The total free radical scavenging activity of the tested extracts and fractions was determined and compared to that of ascorbic and gallic acids as well as trolox by using a slightly modified method of Tuba and Gulcin (30). A 0.3 mM solution of DPPH was prepared in methanol and 500 µL of this solution was added to 1 mL of the extract or fraction at different concentrations (15-240 µg/mL). These solutions were mixed and incubated in the dark for 30 min at room temperature. Then, the absorbance was measured at 517 nm against blank samples lacking scavenger. All assays were carried out in triplicate. The free radical scavenging activity was calculated according to the following formula:

$$\text{Free radical scavening activity (\%)} = \left(1 - \frac{As}{Ac}\right) \times 100$$

where As is the absorbance in the presence of the sample and Ac is the absorbance of the control.

Hydroxyl radical scavenging (HRS) activity (deoxyribose assay)

The HRS activity was measured by studying the competition between deoxyribose and the extracts or fractions for hydroxyl radicals generated by an ascorbate–EDTA–H_2O_2 system (Fenton reaction) as described by Hinnerburg et al. (31). The assay was performed by adding 200 µL of premixed 100 µM $FeCl_3$ and 100 µM EDTA (1 : 1, v/v) solution, 100 µL of 10 mM H_2O_2, 360 µL of 10 mM 2-deoxy-D-ribose, 1 mL of different extract or fraction concentrations (15-240 µg/mL), 400 µL of 50 mM sodium phosphate buffer (pH 7.4) and 100 µL of 1 mM ascorbic acid as per abovementioned sequence. The mixture was incubated at 50°C for 2 h. Thereafter, 1 mL of 2.8% TCA and 1 mL of 1.0% thiobarbituric acid (in 0.025 M NaOH) were added to each tube. The samples were further incubated in a water bath at 50°C for 30 min to develop the pink chromogen. The extent of oxidation was estimated from the absorbance of the solution at 532 nm and the HRS activity of the extract is reported as the percentage inhibition of deoxyribose degradation. All assays were carried out in triplicate. The hydroxyl radical scavenging activity was calculated according to the following formula:

$$\text{Hydroxy radical scavening activity (\%)} = \left(1 - \frac{As}{Ac}\right) \times 100$$

where As is the absorbance in the presence of the sample and Ac is the absorbance of the control.

Nitric oxide (NO) radical scavenging assay

An aqueous solution of sodium nitroprusside at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of Griess reagent. Scavengers of NO compete with oxygen, leading to reduced production of NO (32). The assay was carried out by incubating 500 µL of 10 mM sodium nitroprusside in phosphate buffer (pH 7.4) and 500 µL of different extract or fraction concentrations (15-240 µg/mL) at 37°C for 2 h. The reaction mixture was then mixed with 500 µL of Griess reagent. The absorbance of the chromophore formed during the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylenediamine was read at 546 nm. The percentage inhibition of NO generated was measured in comparison with the absorbance values of a control (10 mM sodium nitroprusside in phosphate buffer). All assays were carried out in triplicate. The free radical scavenging activity was calculated according to the following formula:

$$\text{Nitric oxide radical scavening activity (\%)} = (1 - \frac{As}{Ac}) \times 100$$

where As is the absorbance in the presence of the sample and Ac is the absorbance of the control.

Determination of α-glucosidase inhibitory activity of solvent fractions of the ethanolic extract of the leaves

The α-glucosidase inhibitory activity was determined according to the method described by Ademiluyi and Oboh (33) with slight modifications. Briefly, 250 µL of each fraction or acarbose at different concentrations (30-240 µg/mL) was incubated with 500 µL of 1.0 U/mL α-glucosidase solution in 100 mM phosphate buffer (pH 6.8) at 37°C for 15 min. Thereafter, 250 µL of pNPG solution (5 mM) in 100 mM phosphate buffer (pH 6.8) was added and the mixture was further incubated at 37°C for 20 min. The absorbance of the released p-nitrophenol was measured at 405 nm and the inhibitory activity was expressed as percentage of the control without the inhibitors. All assays were carried out in triplicate. The α-glucosidase inhibitory activity was calculated according to the following formula:

$$\text{α-glucosidase inhibitory activity (\%)} = (1 - \frac{As}{Ac}) \times 100$$

where As is the absorbance in the presence of the sample and Ac is the absorbance of the control.

Determination of α-amylase inhibitory activity of the solvent fractions of the ethanolic extract of the leaves

The α-amylase inhibitory activity was determined according to the method described by Shai et al. (34) with slight modifications. A volume of 250 µL of each fraction or acarbose at different concentrations (30-240 µg/mL) was incubated with 500 µL of porcine pancreatic amylase (2 U/mL) in 100 mM phosphate buffer (pH 6.8) at 37°C for 20 min. Thereafter, 250 µL of 1% starch dissolved in 100 mM phosphate buffer (pH 6.8) was added to the reaction mixture then incubated at 37°C for 1 h. Dinitrosalicylate color reagent (1 mL) was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm and the inhibitory activity was expressed as percentage of a control without the inhibitors. All assays were carried out in triplicate. The α-amylase inhibitory activity was calculated according to the following formula:

$$\text{α-amylase inhibitory activity (\%)} = (1 - \frac{As}{Ac}) \times 100$$

where As is the absorbance in the presence of the sample and Ac is the absorbance of the control.

Mechanism of α-glucosidase and α-amylase inhibition

The most active (aqueous) fraction was subjected to kinetic experiments to determine the type of inhibition exerted on α-glucosidase and α-amylase. The experiment was conducted according to the protocols as described above at a constant concentration of the sample fraction (30 µg/mL) with a variable concentration of substrate as described below. For the α-glucosidase inhibition assay, 0.625-5 mM of pNPG was used and 0.125-1% of starch was used for the α-amylase inhibition assay. The initial rates of the reactions were determined from calibration curves constructed using varying concentrations of p-nitrophenol and maltose for the α-glucosidase and α-amylase inhibition assays, respectively. The initial velocity data obtained were used to construct Lineweaver-Burke's plot to determine the K_M (Michaelis constant) and Vmax (maximum velocity) of the enzyme as well as the Ki (inhibition binding constant) of the inhibitors and the type of inhibition for both enzymes.

Gas chromatography-mass spectrometric (GC-MS) analysis

The most active (aqueous) fraction was further subjected to GC-MS analysis. The analysis was conducted with an Agilent Technologies 6890 GC coupled with (an Agilent) 5973 mass selective detector and driven by an Agilent Chemstation software. A HP-5MS capillary column was used (30 m × 0.25 mm i.d., × 0.25 µm film thickness). The carrier gas

was ultra-pure helium at a flow rate of 0.7 mL/min and a linear velocity of 37 cm/s. The injector temperature was set at 250°C. The initial oven temperature of 60°C was programmed to 280°C at the rate of 10°C/min with a hold time of 3 min. Injections of 2 µL were made in the split mode with a split ratio of 20 : 1. The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230°C, quadrupole temperature 150°C, solvent delay 4 min and scan range 50-700 amu. Compounds were identified by direct comparison of the retention times and mass fragmentation pattern data with those in the National Institute of Standards and Technology (NIST) library.

Statistical analysis

All data are presented as the mean ± SD of triplicates determination. Data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using

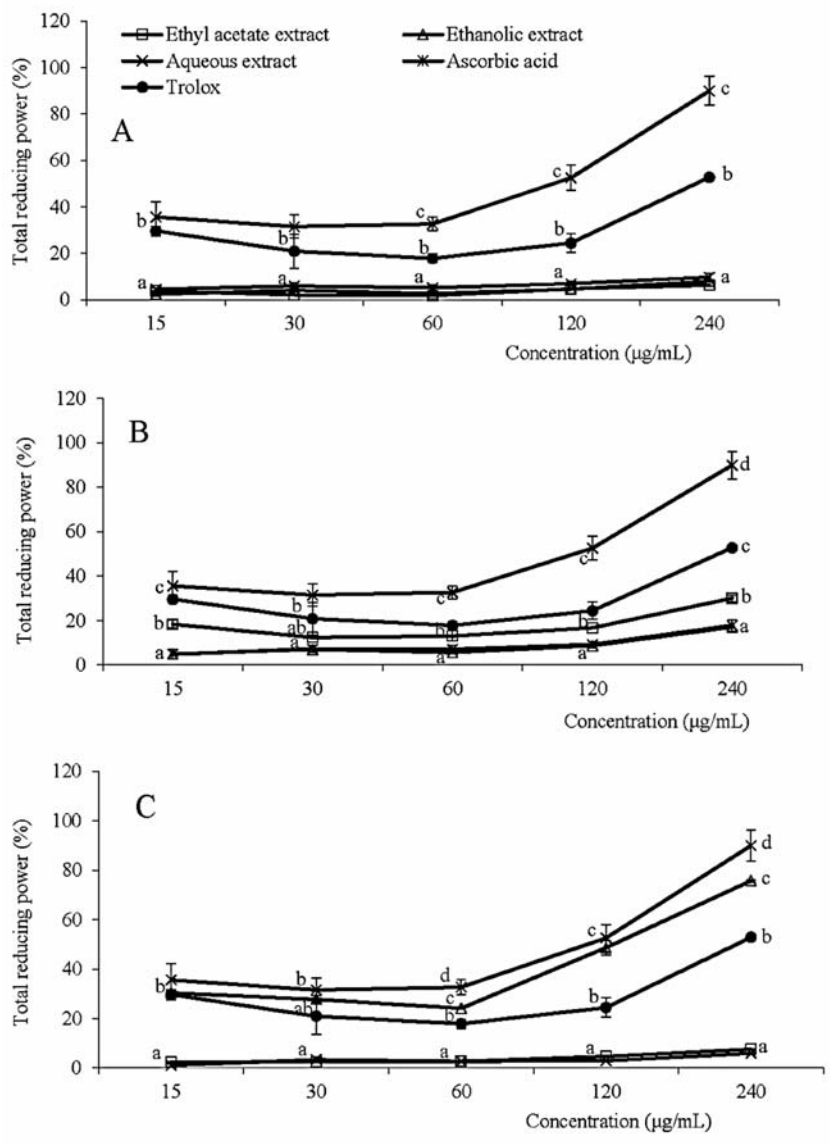


Figure 1. Percentage total reducing power (gallic acid equivalent) of crude extracts of stem bark **(A)**, root **(B)** and leaves **(C)** of *Vitex doniana* parts. Data are presented as the mean ± SD of triplicate determinations. [a-d]Values with different letters presented for a given concentration for each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

Tukey's-HSD multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

RESULTS

The ethanolic extract of the leaves was found to contain a significantly ($p < 0.05$) higher amount of total phenolics than other crude extracts from the different parts of the plant (Table 1). All the stem bark extracts displayed a weak and statistically similar ($p < 0.05$) total reducing power (GAE) compared to ascorbic acid and trolox. For the roots, the ethyl acetate extract had significantly ($p < 0.05$) higher reducing power than other extracts at all con-

Table 1. Percentage yield and total polyphenol concentrations of various solvent fractions of *V. doniana* parts.

Crude extracts %	Yield	Total polyphenol (mg/g GAE)
Stem bark		
Ethyl acetate	0.26	0.86 ± 0.03^{a}
Ethanolic	0.53	4.38 ± 0.38^{c}
Aqueous	0.67	10.65 ± 0.62^{d}
Root		
Ethyl acetate	0.11	17.75 ± 0.64^{e}
Ethanolic	0.42	29.04 ± 3.88^{f}
Aqueous	0.51	19.92 ± 2.15^{e}
Leaves		
Ethyl acetate	0.29	9.76 ± 1.83^{d}
Ethanolic	0.22	50.90 ± 6.06^{g}
Aqueous	0.38	2.62 ± 0.94^{b}

Data are presented as the mean ± SD values of triplicate determinations. $^{a\text{-}g}$Different letters along a column for a given extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 2. IC_{50} values of various extracts of *V. doniana* parts in different anti-oxidative models.

Samples	IC_{50} (µg/mL)		
	DPPH	HRS	NO
Stem bark			
Ethyl acetate	13.70 ± 1.18^{h}*	2.43 ± 0.22^{h}**	298.79 ± 56.05^{e}
Ethanol	170.88 ± 12.40^{f}	7.51 ± 0.90^{b}	4.47 ± 0.13^{j}*
Aqueous	128.93 ± 19.41^{e}	467.54 ± 63.40^{f}	192.10 ± 31.16^{d}
Root			
Ethyl acetate	47.38 ± 7.84^{d}	2.22 ± 0.15^{a}	10.02 ± 1.52^{k}*
Ethanol	2.36 ± 0.34^{b}	37.66 ± 5.15^{c}	549.87 ± 58.84^{f}
Aqueous	1.96 ± 0.16^{ab}	99.66 ± 10.25^{d}	691.22 ± 6.53^{fg}
Leaves			
Ethyl acetate	103.63 ± 5.45^{e}	371.86 ± 23.28^{e}	2.17 ± 0.22^{i}*
Ethanol	2.64 ± 0.76^{b}	2.72 ± 0.60^{a}	45.94 ± 3.04^{b}
Aqueous	6.49 ± 0.10^{g}*	1.37 ± 0.08^{g}*	76.39 ± 6.45^{c}
Ascorbic acid	2.56 ± 0.26^{b}	ND	26.40 ± 6.46^{a}
Gallic acid	1.40 ± 0.43^{a}	ND	1.05 ± 0.23^{h}*
Trolox	8.47 ± 2.88^{c}	3.23 ± 0.49^{a}	629.76 ± 63.98^{f}

Data are presented as the mean ± SD values of triplicate determinations. $^{a\text{-}k}$Different letters along a column for a given sample or standard are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). ND means not determined. *Unit was expressed in mg/mL. **Unit was expressed in g/mL.

Table 3. IC_{50} values of various solvent fractions of ethanolic extract of *V. doniana* leaves in different anti-oxidative models.

Fractions	Total phenolics (mg/g GAE)	IC_{50} (µg/mL)		
		DPPH	HRS	NO
Aqueous	32.57 ± 2.11[a]	67.42 ± 1.54[b]	81.26 ± 1.92	210.29 ± 12.20[a]
Ethyl acetate	24.81 ± 0.68[b]	52.73 ± 0.39[a]	77.93 ± 4.44	697.49 ± 27.71[b]
Dichloromethane	9.27 ± 1.34[c]	92.06 ± 0.44[c]	NIL	NIL
Hexane	NIL	NIL	NIL	NIL

Data are presented as the mean ± SD values of triplicate determinations. [a-c]Different letters along a column for a given fraction are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

Table 4. IC_{50} values for the inhibition of a-glucosidase and a-amylase by various solvent fractions of ethanolic extract of *V. doniana* leaves.

Fractions/standard	IC_{50} (µg/mL)	
	α-glucosidase	α-amylase
Aqueous	41.26 ± 4.25[a]	729.31 ± 145.76[b]
Ethyl acetate	61.98 ± 4.81[b]	1.67 ± 0.10[c]*
Dichloromethane	91.37 ± 5.22[c]	7.27 ± 0.33[d]*
Hexane	NIL	NIL
Acarbose	55.59 ± 5.22[b]	256.66 ± 20.52[a]

Data are presented as the mean ± SD of triplicate determinations. [a-d]Values with different letters within a column for a given parameter are significantly different from each other fractions (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). *Unit was expressed in mg/mL.

centrations. The ethanolic extract of the leaves demonstrated a significantly ($p < 0.05$) higher $Fe^{3+} \rightarrow Fe^{2+}$ reducing ability than all other extracts and trolox (Fig. 1).

The calculated IC_{50} values for the different anti-oxidative models are presented in Table 2. According to the IC_{50} values, the DPPH radical scavenging activities of the various extracts of *V. doniana* parts indicated that the stem bark extracts had weaker free radicals quenching ability than extracts from other parts of the plant (Table 2). Crude ethanolic and aqueous extracts of the root as well as the ethanolic extract of the leaves demonstrated the strongest and statistically similar ($p < 0.05$) free radical scavenging activity among all the crude extracts from the plant. The best HRS activity was observed with ethyl acetate extract of the root ($IC_{50} = 2.22 \pm 0.15$ µg/mL) and the ethanolic extract of the leaves ($IC_{50} = 2.72 \pm 0.60$ µg/mL). Furthermore, in the NO scavenging assay, the ethanolic extract of the leaves showed a significantly higher ($p < 0.05$) scavenging activity towards the generated NO radicals than other extracts from the plant (Table 2). A close analysis of the results obtained from the four different models for anti-oxidative studies indicated that the ethanolic extract of the leaves had the highest anti-oxidative activity that reasonably cuts across all the models used and therefore, was partitioned.

Using solvent-solvent partitioning, hexane, dichloromethane, ethyl acetate and aqueous fractions were obtained. All the fractions possessed reducing power ability but the aqueous fraction displayed the best activity in this model (Fig. 2). However, the results of the DPPH radical scavenging activity showed that the ethyl acetate fraction had a significantly higher ($p < 0.05$) free radical scavenging activity than the aqueous and dichloromethane fractions (Table 3). The results also indicated that the hexane fraction did not contain free radical scavenging phytochemicals. Among the four fractions, only the aqueous and ethyl acetate fractions scavenged hydroxyl and NO radicals. However, the results were not significantly ($p > 0.05$) different from each other in the HRS activity assay whereas the aqueous fraction showed a significantly higher ($p < 0.05$) NO scavenging activity than the ethyl acetate fraction (Table 3).

The α-glucosidase and α-amylase inhibitory activities of the different solvent fractions of the ethanolic extract of the leaves are shown in Figure 3. With the exception of hexane fraction, all other frac-

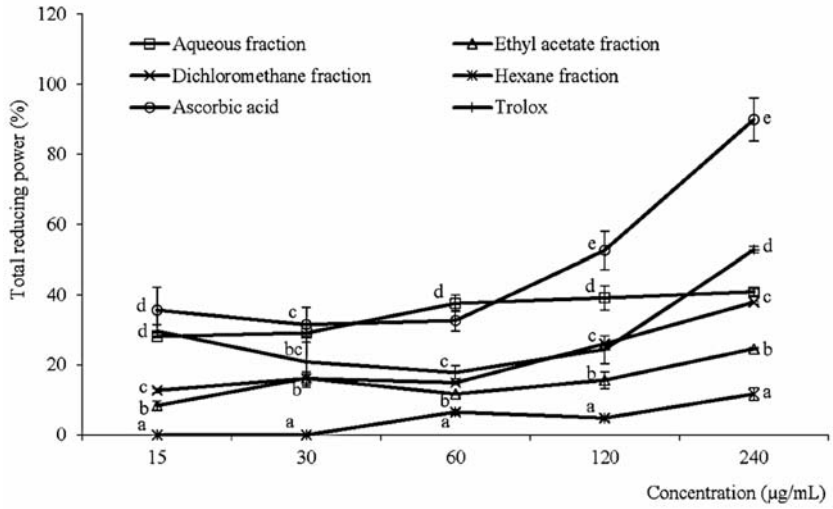


Figure 2. Percentage total reducing power (gallic acid equivalent) of different solvent fractions of ethanolic extract of *V. doniana* leaves. Data are presented as the mean ± SD of triplicate determinations. [a-e]Values with different letters presented for a given concentration are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

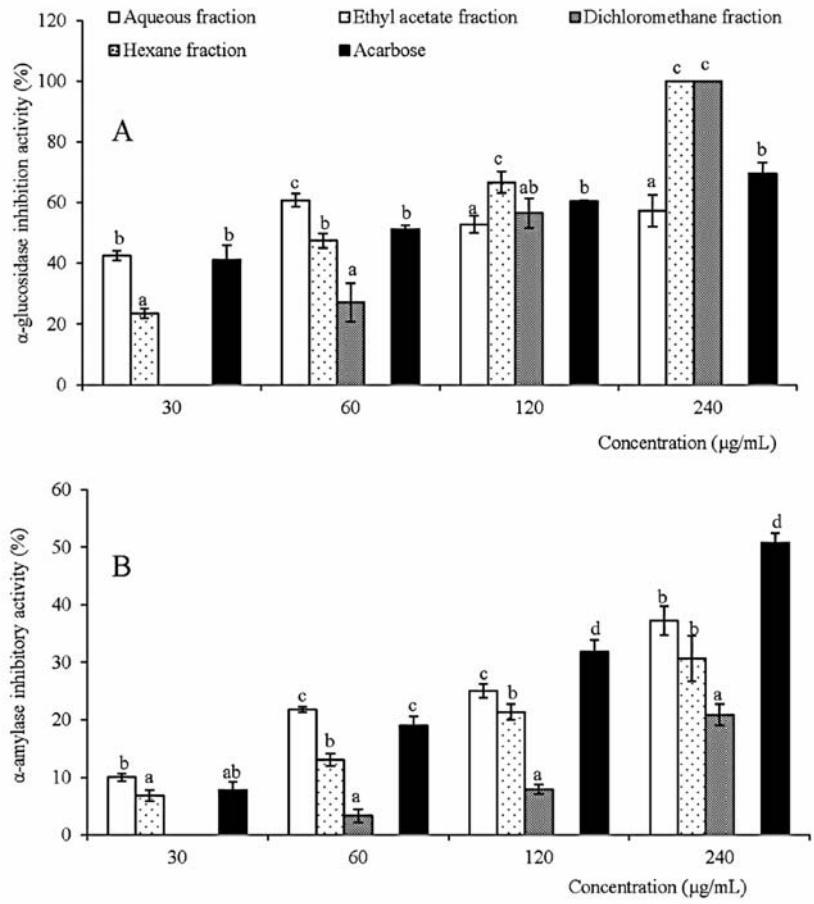


Figure 3. α-Glucosidase (A) and α-amylase (B) inhibitory activities of different solvent fractions of ethanolic extract of *V. doniana* leaves. Data are presented as the mean ± SD of triplicate determinations. [a-d]Values with different letters over the bars for a given concentration are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

tions inhibited α-glucosidase and α-amylase *in vitro* (Fig. 3) but judging from the IC_{50} values, the aqueous fraction also displayed a significantly ($p < 0.05$) higher α-glucosidase and α-amylase inhibitory activities than other fractions (Table 4). Steady state kinetics analysis from the initial velocity studies of α-glucosidase using pNPG as substrate gave a K_M and Vmax values of 2.00 mM and 655.09 µmol/min, respectively, whereas a K_M and Vmax of 0.25 % and 33.70 µmol/min, respectively, were computed for α-amylase using starch as the substrate. Delineation of the type of inhibition exerted by the aqueous fraction revealed that α-glucosidase and α-amylase are non-competitively (Fig. 4) inhibited, albeit at different rates, with Ki values of 5.93 and 167.44 µg/mL, respectively (Table 5).

Based on the above results, the aqueous fraction was subjected to GC-MS analysis in order to identify the components of the fraction. From the experiment, the reasonably identified compounds in the fraction were mainly phenolic compounds such as resorcinol, 4-hydroxybenzoic acid, 3,4,5-trimethoxyphenol and 2,4'-dihydroxychalcone (Fig. 5 and Table 6).

DISCUSSION

Type 2 diabetes is linked to oxidative stress-mediated complications as well as hyperglycemia which are regarded as important underlying factors for the pathogenesis of the disease (35). Thus, agents that can scavenge free radicals and have strong antioxidant as well as α-glucosidase and α-amylase inhibitory properties play a significant role in the treatment and prevention of T2D and related complications. In the present study, the stem bark,

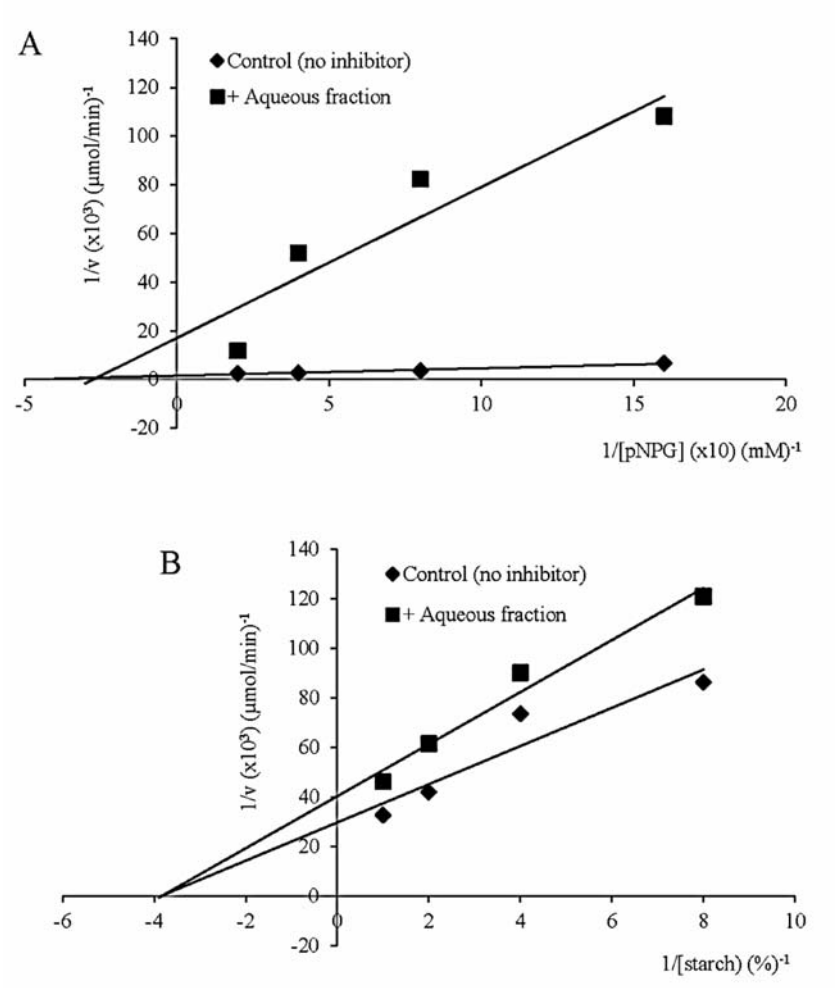


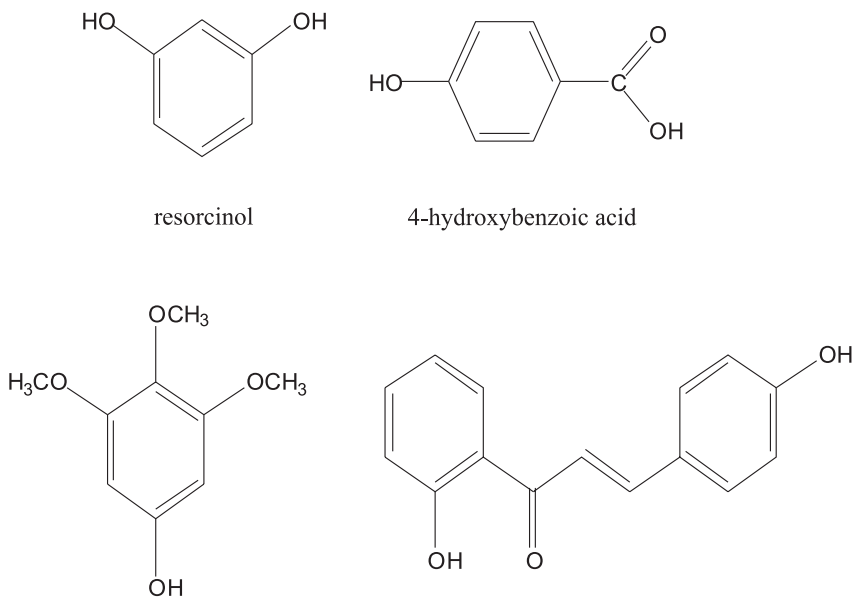
Figure 4. Lineweaver-Burke's plot of α-glucosidase (A) and α-amylase (B) catalyzed reactions in the presence and absence of the aqueous fraction derived from the *V. Doniana* leaves ethanolic extract

root and leaves of different solvent extracts of *V. doniana* were evaluated for their anti-oxidative property which led to the fractionation of the ethanolic extract of the leaves as well as investigation of the anti-oxidative and α-glucosidase and α-amylase inhibitory activity of the fractions.

Four complementary assays were performed to evaluate the anti-oxidative activities of the extracts because a single model cannot give a full evaluation of the anti-oxidative capabilities of the different extracts tested due to the involvement of multiple mechanisms. The total reducing power of a compound serves as a significant indicator of its antioxidant potential. The reductants terminate the free radical chain reaction by donating hydrogen atoms to the radical molecules. Free radicals are known to be a major factor in cellular damages in biological systems and DPPH method has been used to evaluate the free radical scavenging activity of natural antioxidants. On the other hand, hydroxyl radicals are also extremely reactive species capable of damaging any biological molecule found in living systems (36) and NO is an unstable species which reacts with oxygen to generate the reactive nitrite and peroxynitrite anions (37). All these radicals are implicated in the pathogenesis of T2D (38). Findings from this investigation revealed that among the various solvent crude extracts of the different parts of *V. doniana*, the ethanolic extract of the leaves possessed better antioxidant activity having reasonably high scavenging activity toward the various forms of radicals. This observation supports the traditional use of the leaves of *V. doniana* by traditional healers for the management of T2D and other radical pathologies as opposed to the other parts of the plant. Hence, the ethanolic extract of the leaves was selected for further fractionation based on its anti-oxidative activities.

Evaluation of the different fractions from the ethanol extract of the leaves indicated that the aqueous fraction had the best anti-oxidative activity amongst the fractions but the activity was less potent than what was observed for the crude ethanolic extract. This suggests that not all anti-oxidative components were concentrated in the aqueous fraction and other less polar components also contribute to the overall anti-oxidative activity. Nevertheless, at least among the fractions, the aqueous fraction seems to contain the most powerful phytochemical(s) that could be useful therapeutic agents for treating oxidative stress based metabolic disorders including T2D. Moreover, the observed anti-oxidative activity is linked to the highest phenolics content recorded in this fraction because phenolic compounds are the major constituents in plants reported to possess anti-oxidative activity (39).

Analysis of the α-glucosidase inhibitory activity of the fractions also demonstrated that the aque-



resorcinol 4-hydroxybenzoic acid

3,4,5-trimethoxyphenol 2,4'-dihydroxychalcone

Figure 5. Chemical structures of the compounds identified in the aqueous fraction of the ethanolic extract of *V. doniana* leaves

Table 5. Effect of aqueous fraction (60 µg/ml) of ethanolic extract of *V. doniana* leaves on some kinetic parameters of α-glucosidase and α-amylase.

Kinetic parameters	α-Glucosidase		α-Amylase	
	Control	+ aqueous fraction	Control	+ aqueous fraction
K_M	2.00*	2.00*	0.25#	0.25#
V_{max} (µmol/min)	655.09	58.96	33.70	24.81
K_i (µg/mL)	-	5.93	-	167.44

The units for K_M were mM (*) and % (#).

Table 6. Components of the aqueous fraction of *V. doniana* leaves ethanolic extract identified through their mass fragmentation pattern.

Retention time (min)	Compound	Molecular mass
8.05	Resorcinol	110.1
11.43	4-Hydroxybenzoic acid	138.1
12.36	3,4,5-Trimethoxyphenol	184.2
20.94	2,4'-Dihydroxychalcone	240.2

ous fraction contains the most powerful inhibitors against this enzyme which could slow down the breakdown of disaccharides to liberate glucose; thereby reducing glucose absorption from the small intestine (40). This consequently suppresses the postprandial rise in the blood glucose level. The observation could be linked to the high phenolic content recorded in this fraction since polyphenolic fractions from plants have been shown to inhibit α-glucosidase activity and allow for a tight control of blood glucose (41). Indeed, some isolated phenolics have been reported to be the main bioactive anti-diabetic agents of *Brickellia cavanillesii* (42) and *Garcinia mangostana* (43) and the activity was mediated through the inhibition of α-glucosidase. Furthermore, Mai et al. (44) reported a strong positive correlation between polyphenolics content and α-glucosidase inhibitory effects of 28 extracts from Vietnamese edible plants. In the same vein, the aqueous fraction had the highest α-amylase inhibitory activity amongst the fractions. Humans have five α-amylase genes, three encoding salivary α-amylase and two encoding pancreatic α-amylase. Both salivary and pancreatic α-amylases are composed of a single polypeptide chain of 496 amino acids with high degree of amino acid sequence similarity of 97% overall identical residues and 92% in the catalytic domain (8). These similarities suggest that the aqueous fraction could interact with both isozymes through a similar pattern to slow down the breakdown of complex carbohydrates to oligosaccharides, thereby diminishing the effect of carbohydrates consumption on postprandial hyperglycemia. Although the α-amylase inhibitory activity of the aqueous fraction was lower than the reference drug acarbose, but keeping in mind the multiple health benefits of herbal medicines, this finding may provide further scope for investigation.

Kinetic delineation of the α-glucosidase and α-amylase inhibitions indicated that the aqueous fraction inhibited the enzymes in a non-competitive manner suggesting that the bioactive ingredient(s) bind to the enzymes at separate site(s) of the enzyme (rather than the active site) but caused conformational modification at the active site, which prevented the effective binding of the substrates to the enzymes. It could further suggest that the fraction contains some phytochemicals capable of interacting with the α-glucosidase-pNPG and α-amylase-starch complexes (45). Similarly, recent studies have shown that some isolated phenolics such as 6-hydroxyacetyl-5-hydroxy-2,2-dimethyl-2H-chromene, fucofuroeckol A and dioxinodehydroeckol exhibited potent α-glucosidase inhibitions in a non-competitive manner (42, 46). Interestingly, our GC-MS analysis also revealed that the aqueous fraction mainly contains phenolic compounds such as resorcinol, 4-hydroxybenzoic acid and 3,4,5-trimethoxyphenol and 2,4'-dihydroxychalcone. It could thus imply that the observed α-glucosidase and α-amylase inhibitions of this fraction is mediated by these phenolics acting individually or synergistically. The identification of the chalcone in the fraction is also interesting in that chalcones are biosynthetic precursors to the flavonoids and themselves have a range of medicinal properties (47).

The data obtained from this study suggest that the *V. doniana* leaves contain phenolic compounds that could serve as anti-oxidative agents and inhibitors of α-glucosidase and α-amylase which could be exploited for the development of a holistic therapeutic strategy for the control of postprandial

blood glucose levels, T2D and chronic vascular complications. However, the determination of the specific role of each phenolic compound awaits further work on the isolation of these compounds and conducting detailed intervention trials in a rodent model of T2D.

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SECONDARY METABOLITES ISOLATED FROM TWO MEDICINAL PLANT SPECIES, *BRIDELIA MICRANTHA* AND S*IDEROXYLON INERME* AND THEIR ANTIOXIDANT ACTIVITIES

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Abstract: The indigenous medicinal plant species, *Bridelia micrantha* and *Sideroxylon inerme* (both known as 'uMhlalamagwababa' in isiZulu), are used interchangeably by traditional healers in KwaZulu-Natal, South Africa, to treat a variety of ailments due to morphological similarities. In this study, a phytochemical investigation was done on *B. micrantha* and *S. inerme* to determine if they have a chemotaxonomic link. Three pentacyclic triterpenes, one carotenoid and one megastigmane were isolated from *S. inerme*. Two flavonoids and two triterpenes were isolated from *B. micrantha*. These compounds were not previously isolated from these plant species. The antioxidant capacity of the isolated flavonoids was comparable to that of the known antioxidant - ascorbic acid. Profiling of the fruit extracts from both plant species by gas chromatography-mass spectrometry showed different classes of compounds in the extracts. This study corroborates the use of *S. inerme* and *B. micrantha* by traditional healers but does not support their use interchangeably.

Keywords: flavonoids, triterpenes, antioxidants, traditional medicine

Plants have been used throughout history in traditional medicine for the treatment of a wide range of illnesses and diseases. Different parts of plants contain secondary metabolites that are part of the plants defense mechanism to fight against various environmental threats (1) and some of these secondary metabolites are known to have medicinal benefits. The consumption of plants by humans, particularly fruits and vegetables, for their antioxidant value is common practice (2). Natural antioxidants counter the effects of free radicals that promote oxidative damage and are involved in the pathogenesis of many diseases such as cancer, Alzheimer's and diabetes (3, 4). In developing countries, high levels of infectious diseases and the high mortality rate during pregnancy and childbirth in addition to cancer and chronic respiratory diseases account for most deaths (5). Most people in these countries usually turn to natural products for treatment and cure when faced with health-related problems due to accessibility and cost effectiveness.

Bridelia micrantha (known as 'uMhlalamagwababa' in isiZulu) is a fast growing shade tree from the plant family Euphorbiaceae. *B. micrantha* is generally found along the swamp forests in the Eastern Cape, KwaZulu-Natal and Limpopo Province in South Africa. *Sideroxylon inerme* (known as 'uMhlalamagwababa' and 'Amasethole-amhlophe' in isiZulu) is a slow growing, evergreen tree that belongs to the plant family that comprises 1100 species and 53 genera (6). *S. inerme* is found along the coast of South Africa from the Cape Peninsula to Northern Zululand. Both these plant species have similar looking berry-like fruits that ripen in summer, turn black when ripe and produce milk sap. These two plant species are often confused due to morphological similarities, even by traditional healers who use these species interchangeably.

Different parts of *B. micrantha* and *S. inerme* are used traditionally to treat a variety of ailments; the bark is used to treat burns, wounds, venereal diseases, tapeworms, toothache and diarrhea (7). The leaf sap is used to treat sore eyes and the roots are used to treat stomach pains, gastric ulcers and headaches. The herbalists of Western Nigeria use the stem bark to prepare decoctions that are used to prolong pregnancy to full term (8).

Phytochemical studies have not been done on *S. inerme* despite its role in traditional medicine. However, preliminary screening of crude extracts

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has indicated the presence of two phytocompounds, epigallocatechin gallate and procyanidin B (9). Previous studies done on *S. inerme* species have indicated the presence of triterpenoids and sterols which are used as chemotaxonomic markers (10). The main aim of this study was to isolate, characterize and identify the phytocompounds in the leaves, stem bark, roots and fruits of the two plant species, *B. micrantha* and *S. inerme*, to evaluate if they can be used interchangeably in traditional medicine. The isolated compounds were also assessed for their antioxidant activity to determine the plants potential as natural antioxidants.

EXPERIMENTAL

General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker AVANCE III 400 MHz or 600 MHz spectrometer in deuterated chloroform ($CDCl_3$) or deuterated methanol (MeOD) with tetramethylsilane (TMS) as the internal standard. Ultraviolet – Visible (UV-Vis) spectra were obtained on a UV-Vis-NIR Shimadzu UV-3600 spectrophotometer. Infrared (IR) spectra were recorded using a Perkin Elmer Universal ATR spectrometer. Gas chromatography – mass spectrometry (GC-MS) data were recorded on an Agilent GC-MSD apparatus equipped with a DB-5SIL MS (30 m × 0.25 mm i.d., 0.25 µm film thickness) fused silica capillary column. Helium (2 mL/min) was used as a carrier gas and methanol (MeOH) or dichloromethane (DCM) were used to dissolve the samples. The injector was kept at 250^{o}C whilst the transfer line was at 280^{o}C. The column temperature was held at 50^{o}C for 2 min, and then ramped to 280^{o}C at 20^{o}C/min where it was held for 15 min.

Sample collection

The stem bark, leaves, roots and fruits of both plants (*S. inerme* and *B micrantha*) were collected from various sites in KwaZulu-Natal, South Africa. The plants were identified by a taxonomist, Prof A. Nicolas, from the School of Life Sciences, University of KwaZulu-Natal (UKZN), Westville campus and voucher specimens (Shelembe B1 and Shelembe B2) were deposited in the ward herbarium.

Plant materials were dried at room temperature for three weeks, thereafter, ground samples were sequentially extracted exhaustively with hexane, DCM and MeOH. The aqueous MeOH extract was partitioned with equal volumes of DCM followed by ethyl acetate (EtOAc). All extracts were concentrated by rotatory evaporation, dried, and then subjected to column chromatography (Merck Kieselgel 60, 0.063-0.200 mm, 70-230 mesh ASTM). Fractions were profiled using thin layer chromatography (TLC) (Merck silica gel 60, 20 × 20 cm F254 aluminium sheets) and spots were visualized using 10% H_2SO_4 in MeOH and anisaldehyde spray reagent (12.5 mL anisaldehyde and 25 mL H_2SO_4 in 1 L of distilled water).

Screening of plant extracts

Test for steroids and triterpenoids

About 2 mg of dry plant extract was dissolved in acetic anhydride, heated to boiling then cooled. Concentrated H_2SO_4 (1 mL) was added to the mixture and the formation of a green color indicated the presence of steroids whilst a reddish pink color indicated the presence of triterpenoids.

Tests for alkaloids

Dragendorff's test

The formation of an orange or orange to red precipitate on addition of 5 mL of distilled water, 2 M HCl (till a reaction is observed) and 1 mL of Dragendorff's reagent to approximately 2 mg of methanolic extract indicated the presence of alkaloids.

Mayer's test

The formation of a white or pale yellow precipitate on addition of a few drops of Mayer's reagent to approximately 2 mg of methanolic extract indicated the presence of alkaloids.

Test for phenols

The appearance of a bluish green color on addition of $FeCl_3$ to approximately 2 mg of methanolic extract indicated the presence of phenolic compounds.

Test for flavonoids

The appearance of a pink color on addition of 5 mL of 95% ethanol, a few drops of concentrated HCl and 0.5 g of Mg turnings to approximately 2 mg of methanolic extract indicated the presence of flavonoids.

Isolation and purification of compounds from *S. inerme*

The hexane extract (10.66 g) and DCM extract (7.58 g) of leaves were combined due to similar TLC profiles. The combined extract was subjected to column chromatography and separated using a hexane : ethyl acetate step gradient starting with

100% hexane and stepped by 10% to 100% ethyl acetate. Ten mL fractions were collected for each solvent system and the purity of each fraction was examined by TLC. Fractions with similar retention factors (R_f) were combined and concentrated using a rotatory evaporator. Two compounds were isolated, compound **A1** (10.23 mg) in fractions 10-17 and compound **A2** (10.66 mg) in fractions 55-57.

The same procedure was followed for the combined hexane and DCM extracts of the stem bark (11.50 g) and roots (9.5 g). After elution, fractions 35-37 from the stem bark yielded compound **A2** (16.23 mg), fractions 42-43 yielded compound **A3** (12.70 mg) and fractions 56-60 yielded compound **A4** (20.50 mg). After elution, fractions 22-24 from the roots yielded compound **A2** (5.33 mg) and fractions 31-32 yielded compound **A3** (4.99 mg).

Approximately 100 mL of the aqueous methanol extract of the stem bark was partitioned with an equal volume of DCM, in triplicate, to afford the DCM fraction (10.34 g). This fraction was subjected to column chromatography using a hexane : ethyl acetate step gradient, starting with hexane (80%): ethyl acetate (20%) and stepped by 20% to 100% ethyl acetate. Fifty fractions of 20 mL each were collected and fractions 1-26 were combined to yield compound **A5** (23.01 mg).

Isolation and purification of compounds from *B. micrantha*

The MeOH extract of the leaves and stem bark were combined due to similar R_f values on the TLC plate. The combined extract (124 g) was subjected to column chromatography using silica gel as the stationary phase and a hexane : ethyl acetate solvent system starting with hexane (10%) : ethyl acetate (90%) and stepped by 10% to 100% ethyl acetate. Fifty fractions, 5 × 20 mL for each solvent system, were collected and fractions 34-44 were combined and further purified using Sephedex as stationary phase to produce two compounds **B1** (5.12 mg) and **B2** (10.20 mg).

The hexane and DCM extracts from the roots (10.66 g) were combined due to similar R_f values on the TLC plate and subjected to column chromatography. The compounds were eluted with a hexane : ethyl acetate solvent system starting with 100% hexane that was stepped by 20% to 100% ethyl acetate. Sixty fractions, 10 × 20 mL for each solvent system, were collected and profiled using TLC. Fractions with similar profiles were combined and concentrated using a rotatory evaporator. Fractions 9-10 produced compound **B3** (7.55 mg) and fractions 54-55 produced compound **B4** (8.04 mg).

Antioxidant activity

Radical scavenging activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH)

The scavenging ability of the crude extracts of *S. inerme* and *B. micrantha* and compounds **A5**, **B1** and **B2** was measured using the DPPH stable free radical method outlined by Murthy (3) with few modifications. This method is one of the quickest methods of evaluating antioxidant activity because of its widely used index and stability (2). A volume of 300 µL of methanolic solution of crude extract at different concentrations ranging from 15 µg/mL to 1000 µg/mL was mixed with 900 µL of a methanolic solution of DPPH (0.10 mM) and kept in the dark for 30 min. The absorbance was then measured at 517 nm against MeOH and DPPH as a control using a spectrophotometer. Each sample was evaluated in triplicate. The percentage scavenging activity of the radical was determined by the following formula:

$$\% \text{ Scavening} = \left(\frac{A_{Control} - A_{Sample}}{A_{Control}}\right) \times 100$$

A good indicator of the strength of the radical scavenging activity is the IC_{50} value which is the amount of antioxidant necessary to decrease the initial DPPH absorbance by 50% (11). The IC_{50} value was determined graphically by plotting the absorbance of DPPH as a function of sample concentration in µg/mL for the standard and samples.

Radical scavenging activity by hydrogen peroxide

The scavenging activity of the extracts and isolated compounds towards hydrogen peroxide radicals was determined according to the method of Khan et al. (12) with some modifications. A solution of hydrogen peroxide (2 mM) was prepared in phosphate buffer (50 mM, pH 7.4). Plant extracts or isolated compounds of various concentrations (0.4 mL) were added into a test tube containing 0.12 mL of 50 mM phosphate buffer, to which, 3.6 mL of the hydrogen peroxide solution was added then vortexed. The absorbance of hydrogen peroxide at 230 nm was measured after 15 min. Phosphate buffer with hydrogen peroxide was used as a blank and ascorbic acid as a positive control. The percentage scavenging activity of hydrogen peroxide was determined by the following formula:

$$\% \text{ Scavening} = \left(\frac{1_1 - A_S}{A_0}\right) \times 100$$

where A_S is the absorbance with sample and A_0 the absorbance without sample.

Reducing power using the ferric ion reducing antioxidant power (FRAP) assay

Different extracts and isolated compounds of both *B. micrantha* and *S. inerme* in different concen-

trations ranging from 15 to 1000 µg/mL were mixed with 2.5 mL of 0.2 M phosphate buffer and 2.5 mL of 1% potassium ferricyanide. The solutions were incubated at 50°C for 20 min. Thereafter, 2.5 mL of 10% trichloroacetic acid (TCA), 2.5 mL of a previously prepared solution and $FeCl_3$ were added and the solutions were kept aside for 10 min. The absorbance was measured at 700 nm with ascorbic acid as a positive standard.

Statistical analysis

All experimental measurements were carried out in triplicate. Analysis of variance (ANOVA) was performed on data and p-values < 0.05 were considered significant. The means were separated by Tukey's *post-hoc* test to determine significant differences. All statistical analyses were performed using the Statistical Package for the Social Sciences (PASW Statistics, Version 22, IBM Corporation, Cornell, New York).

RESULTS AND DISCUSSION

Phytochemical screening of secondary metabolites

The phytochemical screening of *S. inerme* and *B. micrantha* revealed the presence of the different classes of compounds present in the extracts (Table 1). The results indicate the presence of triterpenes in both plant species. *S. inerme* tested positive for sterols and alkaloids and *B. micrantha* tested positive for flavonoids.

Structure elucidation of compounds from *S. inerme*

Compound **A1** was isolated as a dark yellow oily solid with a mass of 10.23 mg. The IR spectrum showed a broad absorption band at 3462 cm^{-1} (O-H stretch) and 1648 cm^{-1} (C=C). GC-MS data showed molecular ion peak [M^+] at m/z 568.9 which is in agreement with the molecular formula $C_{40}H_{56}O_2$. This data together with ^{1}H-NMR and ^{13}C-NMR data that corresponded with those in the literature (13-15) confirmed compound **A1** to be lutein. Lutein is one of the major carotenoids known for its antioxidant activity. Previous studies have shown that lutein prevents age-related macular degeneration (16-18). Lutein has previously been isolated from many plant species including *Sideroxylon spinosum* of the *Sideroxylon* species (19).

Compound **A2** was isolated as a white crystalline solid with a mass of 32.22 mg. The ^{1}H-NMR spectrum showed characteristic resonances for a pentacyclic triterpene with seven methyl resonances between δ_H 0.7-1.20 ppm but no double bond or carbinylic proton resonances. The ^{13}C-NMR spectrum showed a carbonyl resonance at δ_C 213.14 ppm which was assigned to C-3 due to HMBC correlations. The carbonyl group was further confirmed by the IR spectrum which showed an absorption band at 1714 cm^{-1} (C=O). GC-MS data showed molecular ion peak [M^+] at m/z 426 which is in agreement with the molecular formula $C_{29}H_{48}O$. This data together with NMR data that corresponded with those in the literature (20, 21) confirmed compound **A2** to be friedelin. This compound has previously been isolated from *Maytenus ilicifolia* (22) and *Quercus suber* (1).

Compound **A3** was isolated as a white powder with a mass of 17.69 mg. The ^{1}H-NMR spectrum showed characteristic resonances for sterols at δ_H 5.33 ppm (H-6) and a multiplet at δ_H 3.50 ppm (H-3). The double doublets at δ_H 4.98 ppm (H-21) and δ_H 5.12 ppm (H-20) indicated the sterol to be stig-

Table 1: Qualitative analysis of the phytochemicals in both *S. inerme* and *B. micrantha* extracts (roots (R), bark (B), leaves (L) and fruits (F)).

Test	*S. inerme*					*B. micrantha*				
	MeOH (B)	DCM (B)	DCM (L)	EtOAc (R)	DCM (F)	MeOH (B)	DCM (B)	DCM (L)	EtOAc (R)	DCM (F)
Alkaloids										
Dragendorff's	+	-	-	+	-	-	-	-	-	-
+ Mayer's	+	-	-	+	-	-	-	-	-	-
Phenols	+	-	-	+	-	+	+	-	-	-
Flavonoids	-	-	-	-	-	+	+	+	+	-
Triterpenoids	+	+	-	+	+	+	+	+	+	-
Sterols	-	-	+	-	-	-	-	-	-	+

masterol. The IR spectra showed a broad absorption band at 3432 cm^{-1} (OH group) and 1648 cm^{-1} (C=C). GC-MS data showed molecular ion peak [M^+] at m/z 426 which is in agreement with the molecular formula $C_{29}H_{48}O$. This data together with NMR data that corresponded with those in the literature (20, 23) confirmed compound **A3** to be stigmasterol. Stigmasterol is one of the most ubiquitous phytosterols and has been isolated from numerous plant species (24).

Compound **A4** was isolated as a white powder with a mass of 20.50 mg. The ^{1}H-NMR spectrum showed characteristic resonances for the oleanane-type pentacyclic triterpene with a vinylic proton resonance at δ_H 5.10 ppm (H-12), a carbinylic proton resonance at δ_H 3.20 ppm (H-3), a double doublet at δ_H 2.81 ppm (H-18) and eight methyl resonances. Resonances at δ_C 145.1 and δ_C 121.6, δ_C 139.5 and δ_C 124.3 ppm in the ^{13}C-NMR spectrum were assigned to C-13 and C-12, respectively. This corroborated the presence of a δ^{12}-double bond of the olean-12-ene-type and indicated the presence of a mixture of α and β-amyrin in a ratio of 80 : 20%. The IR spectrum showed a broad absorption band at 3263 cm^{-1} (OH group) and a band at 1459 cm^{-1} (C=C). GC-MS data showed molecular ion peak [M^+] at m/z 426 which is in agreement with the molecular formula $C_{30}H_{50}O$. This data together with NMR data that corresponded with those in the literature (20, 23) confirmed compound **A4** to be a mixture of α and β-amyrin.

Compound **A5** was isolated as a brown oily liquid with a mass of 23.01 mg. The ^{1}H-NMR spectrum showed 3 methyl singlets resonating at δ_H 0.82 ppm (H-13), 1.09 ppm (H-12) and 1.23 ppm (H-10). Vinylic proton resonances were observed at δ_H 6.06 ppm (s, H-4), 5.81 ppm (d, H-7) and 5.80 ppm (dd, H-8). Two doublets resonating at δ_H 2.26 ppm and 2.42 ppm integrated to one proton each. The HSQC spectrum correlated these 2 protons to the same carbon at δ_C 49.80 ppm (C-2). The HSQC spectrum correlated protons resonating at δ_H 4.21, 4.38, 5.80, 5.81 and 6.06 ppm to carbon resonances at δ_C 62.7 ppm (C-11), 68.0 ppm (C-9), 135.7 ppm (C-7), 129.6 ppm (C-8) and 124.6 ppm (C-4), respectively. The ^{13}C-NMR and DEPT spectra showed 3 quaternary carbons resonances at δ_C 41 (C-1), δ_C 78 (C-6) and δ_C 162 (C-5) ppm; 4 olefinic carbon resonances between δ_C 124 and 163 ppm, of which 3 were methine resonances (C-4, C-7, C-8) and one was a quaternary carbon resonance (C-5); a methylene at δ_C 62.5 ppm (C-11) bearing a hydroxyl group that correlated with the proton at δ_H 4.22 ppm and a methine at δ_C 68.0 ppm (C-9) that correlated with the doublet at δ_H 4.37 ppm. GC-MS spectral data showed molecular ion peak [M^+] at *m*/*z* 239 and base peak at *m*/*z* 207 which was in agreement with molecular formula $C_{13}H_{20}O_4$. The fragmentation pattern as well as spectroscopic and physical data of compound **A5** were consistent with those reported in literature for apocynol B (25) confirming compound **A5** to be apocynol B.

Apocynol B has not previously been isolated from plant extracts, but was obtained from enzymatic hydrolysis of apocynoside II. Apocynoside II was isolated from the roasted leaves of *Apocynum venetum* L. by Murakami and co-workers (25). Biological testing has not been done on these compounds, but compounds from this class are known to possess anti-obesity activity. They are good inhibitors of pancreatic lipase and adiposity differentiation which hydrolyzes and stores fats in the small intestines (26).

Structure elucidation of compounds from *B. micrantha*

Compound **B1** was isolated as a yellow solid with a mass of 5.12 mg and compound **B2** was isolated as a yellow powder with a mass of 10.20 mg. The ^{1}H-NMR spectra of compounds **B1** and **B2** exhibited characteristic resonances for a flavonol. The aromatic region exhibited the ABX system with protons resonating at δ_H 7.76 ppm (H-2'), δ_C ppm 7.66 (H-6') and δ_C 6.91 ppm (H-5') due to disubstitution of ring B and a meta-coupled pattern with protons resonating at δ_H 6.41 ppm (H-8) and δ_H 6.21 ppm (H-6) due to disubstitution of ring A. The ^{13}C-NMR spectra indicated the presence of 15 and 21 carbon signals for compounds **B1** and **B2**, respectively, which resolved 5 methylene and 10 quaternary carbon resonances in the DEPT90 and 135 experiments for compound **B1**. For compound **B2**, the resonance at δ_H 4.9 ppm (H-1'') due to the anomeric proton indicated the presence of a glycosidic linkage. This was confirmed by resonances between δ_H 3.2a3.7 ppm (H-2''- H-6''). The carbon resonance at δ_C 62.5 ppm (C-6'') was shown to be a methylene indicating that the sugar is a glucose attached at position 3 as confirmed by HSQC and HMBC correlations.

The [M]$^+$ ion at *m*/*z* 302 for compound **B1** is in agreement with the molecular formula $C_{15}H_{10}O_7$ for quercetin and the [M]$^+$ ion at *m*/*z* 465 for compound **B2** is in agreement with the molecular formula $C_{21}H_{20}O_{12}$ for quercetin-3-*O*-glucoside. The physical and spectroscopic data for compounds **B1** and **B2** matched those published in the literature (27, 28) therefore, these compounds were identified as the

aglycone, quercetin and its glycoside, quercetin-3-*O*-glucoside, respectively.

Compound **B3** was isolated as a white amorphous powder with a mass of 7.55 mg. The ^{1}H-NMR spectrum showed characteristic resonances for the oleanane-type pentacyclic triterpene with a vinylic proton resonance at δ_H 5.23 ppm (H-12), a carbinylic proton resonance at δ_H 3.18 ppm (H-3), a double doublet at δ_H 2.21 ppm (H-18) and seven methyl resonances. The ^{13}C-NMR spectrum showed resonances at δ_C 126.9 ppm (C-12), δ_C 139.6 ppm (C-13) and δ_C 180.8 ppm (C-28), which corroborated the presence of a δ^{12}-double bond and carboxylic acid functionality. The IR spectrum showed a broad absorption band at 3462 cm^{-1} (OH group) and a band at 1703 cm^{-1} (C=O). GC-MS data showed molecular ion peak [M$^+$] at m/z 456 which is in agreement with the molecular formula $C_{30}H_{48}O_3$. These data together with NMR data that corresponded with those in the literature (20, 23) confirmed compound **B3** to be oleanolic acid.

Compound **B4** was isolated as a white amorphous powder with a mass of 8.04 mg. The ^{1}H-NMR spectrum showed characteristic resonances for the

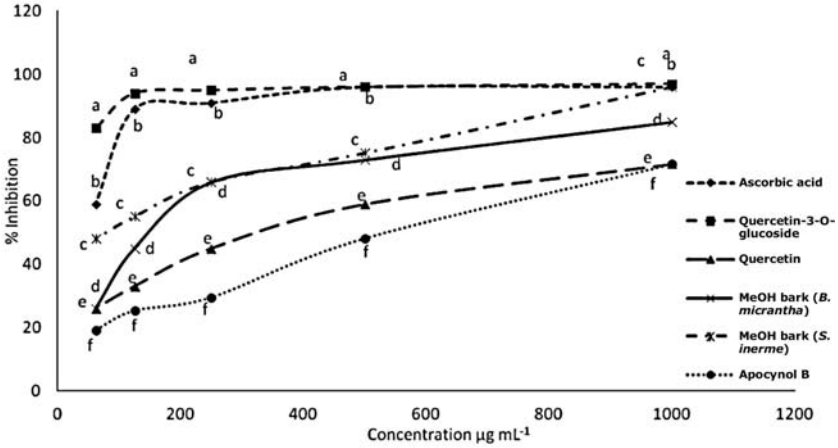

Figure 1. Results of the antioxidant activity of the methanolic extracts and compounds isolated from *S. inerme* and *B. micrantha* as determined by the DPPH method. Different letters indicate mean separation by Tuckey's *post-hoc* test at the 5% level

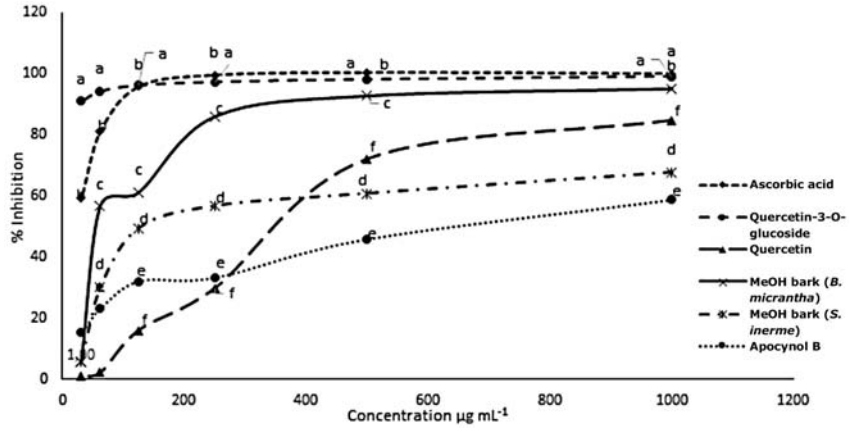

Figure 2. Results of the antioxidant activity of the methanolic extracts and compounds isolated from *S. inerme* and *B. micrantha* as determined by the hydrogen peroxide method. Different letters indicate mean separation by Tuckey's *post-hoc* test at the 5% level

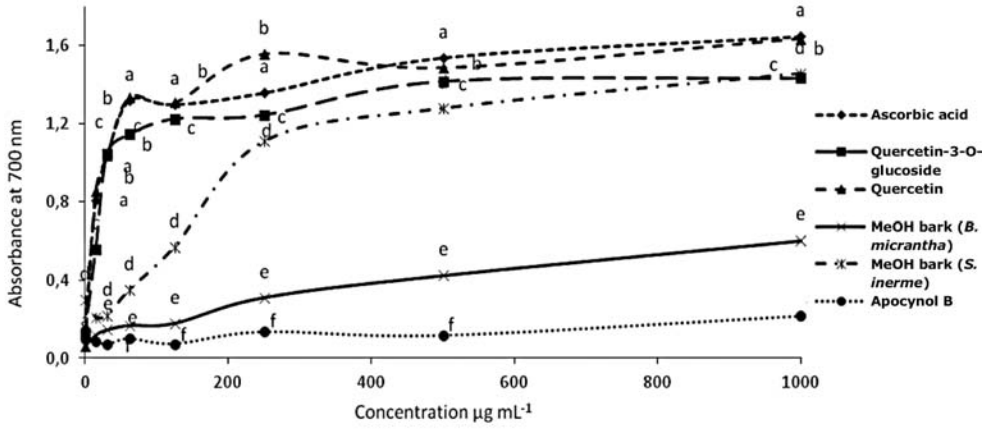

Figure 3. Reducing power of the methanolic extracts and compounds isolated from *S. inerme* and *B. micrantha* as determined by the FRAP method. Different letters indicate mean separation by Tuckey's *post-hoc* test at the 5% level

oleanane-type pentacyclic triterpene with the presence of a Δ^{12}-double bond. Spectral data for compound **B4** was similar to that of compound **B3**, except for the signals corresponding to the lactone ring at C-21. The IR absorption band at 1766 cm^{-1} and the peak at δ_C 181.8 ppm in the ^{13}C-NMR spectrum showed the presence of a γ-lactone ring. The formation of the lactone ring with the carboxyl group at C-17 was confirmed by the key HMBC correlation at δ_H 4.21 ppm (H-21) with the carboxyl carbon at δ 181.8 ppm (C-28). GC-MS data showed molecular ion peak [M^+] at m/z 454.34 which is in agreement with the molecular formula $C_{30}H_{46}O_3$. These data together with NMR data that corresponded with those in the literature (20, 29) confirmed compound **B4** to be acacic acid lactone.

Antioxidant activity

The antioxidant activity of the crude MeOH extracts of the stem bark of *B. micrantha* and *S. inerme* and selected isolated compounds were measured by three assays namely DPPH, hydrogen peroxide and FRAP. For both plant species, the scavenging effect of the extracts and tested phytocompounds increased with increasing concentrations (Figs. 1 and 2). The higher antioxidant activity of the *S. inerme* stem bark extract could be attributed to the stabilizing effect of apocynol B that has four hydroxyl groups capable of donating protons thereby reducing the DPPH radical. The higher antioxidant activity of the *B. micrantha* stem bark extract could be attributed to the two flavonoids present in this extract. Of the two flavonoids, the activity of the glycoside for all concentrations is much higher than the aglycone and even ascorbic acid. This indicates that the sugar moiety enhances the antioxidant activity of the compound. It would appear that the effect of the compounds in the extract is additive as seen by the decreased antioxidant activity in the extract when compared to the individual activities especially that of quercetin-3-*O*-glucoside. The IC_{50} values for the MeOH extract of the stem bark of *S. inerme* and *B. micrantha* were approximately 45 µg/mL and 150 µg/mL, respectively, and that for the standard ascorbic acid was 41 µg/mL indicating the high antioxidant potential of the plant, especially the stem bark. This was further verified by measuring the reducing power of the tested extracts and phytocompounds using the FRAP assay (Fig. 3).

The fruits of both plant species also exhibited good free radical scavenging activity (IC_{50} values 800 µg/mL), therefore consuming fruits from these plants may be beneficial to human health in minimizing oxidative stress.

GC-MS profiling of fruit extracts from *S. inerme* and *B. micrantha*

GC-MS profiling led to the identification of 8 compounds from the fruit extracts of *B. micrantha* and *S. inerme*. The structures and names for each compound were obtained from the National Institute of Standards and Technology (NIST) library (30). The investigation of *B. micrantha* revealed the presence of mostly sterols including ergosterol, cycloartenol, cycloartenol acetate and stigmast-8(14)-en-3-ol while that of *S. inerme* revealed the presence of

mostly triterpenes including fridelin, α-amyrin, β-amyrin and α-amyrin acetate. Triterpenes and plant sterols have previously been reported by many researchers to possess pharmacological activities such as anti-inflammatory, antioxidant, antiviral, antibacterial, gastro-protective, cytotoxic and anti-ulcerogenic activities (20, 31-33). GC-MS profiling indicates that the fruits of these two plant species possess different secondary metabolites.

CONCLUSIONS

The phytochemical investigation lead to the isolation of three pentacyclic triterpenes, one carotenoid and one megastigmane from *S. inerme* as well as two flavonoids and two triterpenes from *B. micrantha*. These compounds were not previously isolated from these plants. The MeOH extract of the stem bark of *S. inerme* appeared to have high antioxidant activity which was similar to that of ascorbic acid at higher concentrations whilst the antioxidant activity of the glycoside isolated from *B. micrantha* had the highest antioxidant activity, even higher than that of ascorbic acid. The phytochemical screening indicated differences in the classes of compounds found in the two plant species. Also, the secondary metabolites isolated from the stem bark, leaves and roots or those identified in fruits using GC-MS analysis of these two plant species were different. This shows that these two plant species, although morphologically similar, do not have chemotaxonomic similarities. This study corroborates the use of *S. inerme* and *B. micrantha* by traditional healers but does not support their use interchangeably.

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PHARMACEUTICAL TECHNOLOGY

A STUDY OF COMPRESSION PROCESS AND PROPERTIES OF TABLETS WITH MICROCRYSTALLINE CELLULOSE AND COLLOIDAL SILICON DIOXIDE

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Abstract: This paper compares the compressibility and properties of tablets from Prosolv SMCC 90 and a mixture of Avicel PH-102 and colloidal silicon dioxide with a different specific surface. The effect of an addition of the lubricant magnesium stearate on these parameters under varying conditions of mixing and the homogeneity of the lubricant in the mixtures are also examined. Compressibility is evaluated by means of the energy balance of the compression process; the examined properties of tablets are tensile strength and disintegration time. The total energy of compression was increased with compression force, the highest being in Prosolv SMCC 90. Its values did not differ for differing conditions of mixing with the lubricant. Plasticity was slightly decreased with compression force and in the mixture with magnesium stearate it was not influenced by the conditions of mixing. Tablets made from Prosolv SMCC 90 and Avicel PH-102 were stronger than those from the mixtures from Avicel PH-102 and both types of Aerosil. The addition of magnesium stearate markedly decreased the strength of tablets from Avicel PH-102. An increase in the period and frequency of mixing with the lubricant resulted in a further decrease in strength. Disintegration time was longer in tablets from Avicel PH-102 and Prosolv SMCC 90, and it was further prolonged by an addition of magnesium stearate.

Keywords: Avicel PH-102, Prosolv SMCC 90, colloidal silicon dioxide, energy profile of compression, magnesium stearate, tensile strength of tablets, disintegration time of tablets

A combination of microcrystalline cellulose (MCC) Avicel PH-102 and colloidal silicon dioxide is a common combination of these excipients in a directly compressible tableting material. In this tableting material, Avicel PH-102 functions as a dry binder and colloidal silicon dioxide as a glidant (1, 2). Both substances are also components of the co-processed dry binder Prosolv SMCC 90, composed of 2% of colloidal silicon dioxide and 98% of microcrystalline cellulose (3, 4). This co-processed dry binder exerts excellent compressibility and also lower sensitivity to additions of lubricants due to the presence of colloidal silicon dioxide, which on the surface of MCC blocks the binding sites for the lubricant and thus decreases the effect of the lubricant on the binding of MCC (5-7). The substance is prepared using the method of spray drying, in which silicon oxide simply adheres to the surface of microcrystalline cellulose, where it is deagglomerated and uniformly distributed. Deagglomeration does not take place in a physical mixture of both substances, it contains large agglomerates of silicon dioxide (8). Colloidal silicon dioxide is mostly known under the trademark Aerosil. It exists in several types, which differ in their specific surfaces, which vary in a range of 100-380 m^2/g. The most common type employed as a glidant is Aerosil 200 of the specific surface of 200 m^2/g (9).

The paper aimed to compare the compressibility of tableting materials and properties of tablets with Avicel PH-102 and Aerosil with different specific surfaces *versus* Prosolv SMCC 90. It also examined the effect of the addition of the lubricant magnesium stearate on the same parameters under varying conditions of mixing. Compressibility was evaluated by means of the energy balance of compression process, the examined properties of tablets were tensile strength and disintegration time.

EXPERIMENTAL

Materials

The study employed microcrystalline cellulose Avicel® PH-102 (FMC Corporation, USA), silici-

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fied microcrystalline cellulose Prosolv® SMCC 90 (JRS PHARMA, Germany), colloidal silicon oxide in two types, i.e., Aerosil® 200 and Aerosil® 255 (Evonic Industries AG, Germany). The lubricant employed was magnesium stearate (Acros Organics, USA).

Preparation of tableting materials

The study employed the following tableting materials:

- Avicel PH-102
- Prosolv SMCC 90
- Avicel PH-102 + 2% Aerosil 200
- Avicel PH-102 + 2% Aerosil 255
- Avicel PH-102 + 1% magnesium stearate
- Prosolv SMCC 90 + 1% magnesium stearate
- Avicel PH-102 + 2% Aerosil 200 + 1% magnesium stearate
- Avicel PH-102 + 2% Aerosil 255 + 1% magnesium stearate

For the first stage of the study, mixtures of Avicel PH-102 with 2% of Aerosil 200 and Aerosil 255 were prepared. Colloidal silicon dioxide was premixed with Avicel PH-102 in a melamine mortar for 1 min and subsequently the mixtures were mixed in a mixing cube KB 15S (Erweka GmbH, Germany) for 5 min. The rate of rotation of the cube was 17 rev/min, the amount of prepared mixtures was 30 g.

For the second stage of the study, the lubricant magnesium stearate in a concentration of 1% was added to the mixtures of Avicel PH-102 with colloidal silicon dioxide under three different conditions of mixing, i.e., 2.5 min 17 rev/min, 5 min 17 rev/min and 2.5 min 34 rev/min. The mixture with magnesium stearate under the same conditions of mixing was prepared also with Avicel PH-102 and Prosolv SMCC 90. The amount of tableting materials prepared in the same manner was 20 g.

Preparation of tablets and energy evaluation of compression process

Tablets were compressed using the material testing equipment T1-FRO 50 TH.A1K Zwick/Roell (Zwick GmbH&Co., Germany) by means of a special die with a lower and an upper punch. The rate of compaction was 40 mm/min, pre-load was 2 N, and the rate of pre-load 2 mm/s. The tablets were of cylindrical shape without facets of a diameter of 13 mm and weight of 0.5 ± 0.0010 g. Compression forces for tableting materials without magnesium stearate were 2.5, 3 and 3.5 kN. Tableting materials with magnesium stearate were compacted using a compression force of 3.5 kN. At each compression force 16 tablets were compacted. In 10 tablets the computer program testXpert V 9.01 simultaneously recorded the energy process of compression by means of the "force-displacement" record and numerically evaluated the energy balance of compression, i.e., the energy consumed for friction E_1, energy accumulated by the tablet after compression E_2, and the energy released during decompression E_3, total energy E_{max}, which is the sum total of all energies, and plasticity (10).

Measurement of the tensile strength of tablets

The tensile strength of tablets was measured in 10 tablets more than 24 h after compression. Measurements were performed using a Schleuniger apparatus ((Dr. Schleuniger Pharmatron AG, Switzerland), which measures the diameter and height of tablets with a precision of 0.01 mm and destruction force in N. The tensile strength of tablets was subsequently calculated according to the equation [1] (11):

$$P = 2 \times F/(\pi \times d \times h) \quad [1]$$

where P is tensile strength of tablets in MPa, F is destruction force in N, d is the diameter of tablets in mm, h is the height of tablets in mm.

Measurement of the disintegration time of tablets

Disintegration time was always measured in 6 tablets at each compression force at least 24 h after compaction. The measurements were made on a device for testing the disintegration time of tablets Erweka ZT 301 (Erweka GmbH, Hausenstamm, Germany) using the method described in the European Pharmacopoeia 8th edition (12). The test was carried out without discs in the medium of purified water tempered for 37 ± 1°C. The tablets were considered disintegrated at the moment when on the net of the tube there was no remainder.

Testing of homogeneity of tableting materials

Homogeneity of mixtures with 1% of magnesium stearate obtained under different mixing conditions was tested on a FTIR spectrometer Nicolet iN10 MX (Thermo, USA), by means of which maps of distribution of the lubricant in the tablet were obtained. In each mixture, two tablets were compressed using a compression force of 3.5 kN. Tablets were cut using a surgeon blade. No polishing was applied prior to measurement. The cut tablets were placed on a microscopic glass cross-section side up and analyzed. Each spectrum was accumulated by acquisition of 1 scan. The image was acquired from an area of 100 × 100 pts. with 30 micrometer steps.

Statistical processing of results

The results of tensile strengths and disintegration time of tablets were statistically processed by means of the computer program Excel. The values of energies and plasticity were statistically processed by the computer programme testXpert V 9.0 directly during compaction. In the case of similar significance of values, unpaired *t*-test at a level of significance of 0.05 was employed.

RESULTS AND DISCUSSION

The study aimed to compare the compressibility and properties of tablets from silicified microcrystalline cellulose Prosolv SMCC 90 with physical mixtures of microcrystalline cellulose with varying types of colloidal silicon dioxide in a concentration of 2%. The employed microcrystalline cellulose was Avicel PH®-102, colloidal silicon dioxide was used in two types of Aerosil® 200 and Aerosil® 255. Avicel PH-102 alone was also evaluated. Compressibility was tested by means of the energy profile of compression, i.e., by means of calculation of total energy E_{max}, energy for friction E_1, energy accumulated by the tablet after compression E_2, energy of decompression E_3, and plasticity. Another parameter related to compressibility is also tensile strength, which was tested in completed tablets together with disintegration time. In the first stage of the study, these parameters were evaluated at three compression forces of 2.5, 3 and 3.5 kN. Compression forces were selected in such a way that the resultant strength may oscillate as close as possible to the optimal range of strength of 0.56–1.11 MPa (13). In the second stage of the study, the effect of an addition of 1% of magnesium stearate was tested under three different conditions of mixing (2.5 min and 17 rev/min, 5 min and 17 rev/min, 2.5 min and 34 rev/min) for the above-mentioned parameters of tableting materials and tablets. In addition, in these mixtures an analysis of homogeneity of the distribution of the lubricant was performed by means of a FTIR spectrophotometer Nicolet iN10 MX.

Evaluation of tableting materials without magnesium stearate

Energy profile of compression

The results of the energy profile of compression of tableting materials without a lubricant are shown in Table 1. The total energy of compression E_{max} increases with compression force and is the highest in the case of Prosolv SMCC 90 excepting the compression force of 2.5 kN, where there is no marked difference in the values between the tableting materials. As the total energy is given by the sum total of the energy for friction E_1, energy accumulated by the tablet after compression E_2 and the energy for friction E_3, it is evident from these energies that the comparison of their values is determined primarily by the energy for friction E_1, in which the course of values is analogous. The values of the energy accumulated by the tablet after compression E_2 increase again with the compression

Table 1. Values of energy profile of compression and plasticity: tableting materials without magnesium stearate.

Tableting material	CF [kN]	E_{max} ± SD [J]	E_1 ± SD [J]	E_2 ± SD [J]	E_3 ± SD [J]	PI ± SD [%]
Prosolv SMCC 90	2.5	5.68 ± 0.12	2.030 ± 0.101	3.414 ± 0.034	0.239 ± 0.010	93.44 ± 0.31
	3	11.26 ± 0.13	6.967 ± 0.158	3.998 ± 0.005	0.299 ± 0.005	93.04 ± 0.13
	3.5	13.57 ± 0.12	8.593 ± 0.108	4.609 ± 0.032	0.363 ± 0.006	92.70 ± 0.10
Av PH102	2.5	5.52 ± 0.06	2.086 ± 0.066	3.190 ± 0.019	0.245 ± 0.003	92.87 ± 0.10
	3	7.01 ± 0.07	2.880 ± 0.084	3.824 ± 0.025	0.304 ± 0.006	92.63 ± 0.12
	3.5	9.04 ± 0.14	4.245 ± 0.146	4.423 ± 0.028	0.370 ± 0.005	92.28 ± 0.10
Av PH102 + A 200	2.5	5.35 ± 0.09	2.075 ± 0.068	3.050 ± 0.028	0.221 ± 0.009	93.24 ± 0.24
	3	7.14 ± 0.16	2.732 ± 0.124	4.127 ± 0.062	0.276 ± 0.004	93.73 ± 0.15
	3.5	8.76 ± 0.25	3.577 ± 0.205	4.850 ± 0.061	0.330 ± 0.003	93.64 ± 0.09
Av PH102 + A 255	2.5	5.53 ± 0.08	1.867 ± 0.064	3.449 ± 0.020	0.212 ± 0.003	94.22 ± 0.10
	3	6.77 ± 0.11	2.326 ± 0.092	4.174 ± 0.045	0.268 ± 0.003	93.97 ± 0.10
	3.5	8.58 ± 0.23	3.533 ± 0.166	4.716 ± 0.098	0.333 ± 0.008	93.40 ± 0.25

Explanations: Av PH102 - Avicel PH 102; A 200 - Aerosil 200; A 255 - Aerosil 255; CF - compression force; E_{max} - total energy; E_1 - energy of friction; E_2 - energy accumulated by the tablet; E_3 - energy of decompression; Pl - plasticity; SD - standard deviation

force. Slightly higher values are shown by the mixtures of Avicel PH-102 with both types of Aerosil, the lowest values being shown by Avicel PH-102 alone. In the energy of decompression E_3, which also increases with compression force, the result is a contrary one, because the lowest values are shown by the mixtures of Avicel PH-102 with both types of Aerosil. The final parameter under evaluation is plasticity, which as a result of a decrease in the pores in the tablet is slightly decreased with compression force. The lowest values of plasticity are shown by Avicel PH-102 alone, followed by Prosolv SMCC 90, and the highest values are recorded for mixtures of Avicel PH-102 with both types of Aerosil. However, it is necessary to state that the differences in the values are in no way marked.

Tensile strength and disintegration time of tablets

The results of tablet strength are shown in Figure 1. Tensile strength increases with compression force in all tableting materials. Its higher values are shown by Avicel PH-102 and Prosolv SMCC 90, while at the compression force of 2.5 kN a higher value is found in Prosolv SMCC 90, in the compression force of 3 kN the value equals to that of Avicel PH-102 and in the compression force of 3.5 kN the value is higher for Avicel PH-102. It means that in Avicel PH-102 binding capacity is increased

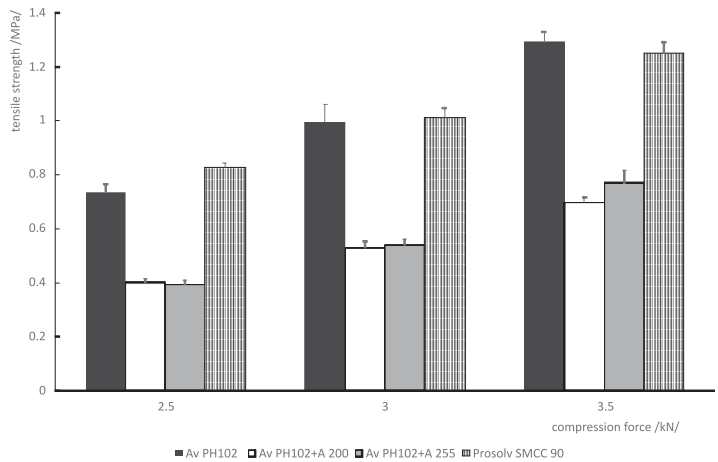

Figure 1. Tensile strength of tablets in function of compression force: tableting materials without magnesium stearate

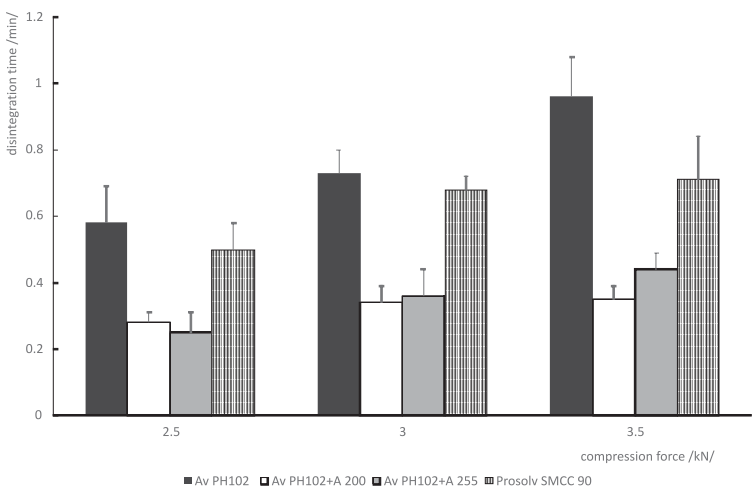

Figure 2. Disintegration time of tablets in function of compression force: tableting materials without magnesium stearate

Table 2. Values of energy profile of compression and plasticity at the compression force of 3.5 kN: tableting materials with magnesium stearate.

Tableting material	Mixing min; rev	E_{max} ± SD [J]	E_1 ± SD [J]	E_2 ± SD [J]	E_3 ± SD [J]	PI ± SD [%]
Prosolv SMCC 90 + 1% st	2.5; 17	8.73 ± 0.09	3.802 ± 0.087	4.549 ± 0.028	0.382 ± 0.007	92.26 ± 0.09
	5; 17	8.79 ± 0.09	3.857 ± 0.093	4.550 ± 0.021	0.383 ± 0.004	92.23 ± 0.08
	2.5; 34	9.19 ± 0.14	4.255 ± 0.130	4.552 ± 0.034	0.385 ± 0.004	92.20 ± 0.10
Av PH102 + 1% st	2.5; 17	8.27 ± 0.08	3.522 ± 0.106	4.371 ± 0.004	0.374 ± 0.004	92.11 ± 0.11
	5; 17	7.86 ± 0.10	3.236 ± 0.104	4.251 ± 0.023	0.375 ± 0.006	91.88 ± 0.10
	2.5; 34	7.84 ± 0.17	3.259 ± 0.151	4.214 ± 0.042	0.372 ± 0.004	91.89 ± 0.09
Av PH102 + A 200 + 1% st	2.5; 17	9.56 ± 0.12	4.475 ± 0.126	4.703 ± 0.023	0.378 ± 0.004	92.57 ± 0.05
	5; 17	7.64 ± 0.09	3.021 ± 0.083	4.253 ± 0.015	0.370 ± 0.003	92.00 ± 0.06
	2.5; 34	7.96 ± 0.07	3.319 ± 0.080	4.272 ± 0.013	0.372 ± 0.004	92.00 ± 0.09
Av PH102 + A 255 + 1% st	2.5; 17	9.39 ± 0.09	4.334 ± 0.092	4.681 ± 0.017	0.376 ± 0.004	92.57 ± 0.06
	5; 17	9.52 ± 0.16	4.511 ± 0.155	4.638 ± 0.020	0.374 ± 0.004	92.54 ± 0.05
	2.5; 34	9.50 ± 0.12	4.483 ± 0.115	4.645 ± 0.028	0.375 ± 0.003	92.53 ± 0.08

Explanations: Av PH102 - Avicel PH 102; A 200 - Aerosil 200; A 255 - Aerosil 255; CF - compression force; E_{max} - total energy; E_1 - energy of friction; E_2 - energy accumulated by the tablet; E_3 - energy of decompression; Pl - plasticity; SD - standard deviation

with increasing force, whereas in Prosolv SMCC 90 it is already high enough at the lowest compression force employed. Mixtures of Avicel PH-102 with both types of Aerosil show, under the employed compression forces, a markedly lower strength of tablets, and between their values there is no statistically significant difference except at the compression force 3.5 kN, where only at this compression force the strength of tablets vacillates slightly above the lower limit of the optimal strength of tablets (0.56 MPa) (13).

The disintegration time of tablets against compression force is shown in Figure 2. The disintegration time of tablets from all tableting materials increases with compression force, this increase being more marked in Avicel PH-102 and Prosolv SMCC 90, in which the values of disintegration time are higher. The longest disintegration time is shown by Avicel PH-102, a somehow shorter one due to the slightly disintegrating effect of colloidal silicon dioxide Prosolv SMCC 90 (3), and the shortest disintegration time is shown by the tablets made from the mixtures of Avicel PH-102 and both types of Aerosil resulting from low strength of tablets.

Evaluation of tableting materials with magnesium stearate

The lubricant magnesium stearate in a concentration of 1% was added to the tableting materials tested in the first stage of the study under three conditions of mixing. A twofold mixing time and twofold mixing rate were tested. The employed conditions of mixing were as follows: 2.5 min 17 rev/min; 5 min 17 rev/min; 2.5 min 34 rev/ min. The employed compression force was 3.5 kN.

Energy profile of compression

The results of the energy profile of compression of tableting materials with a lubricant are shown in Table 2. The values of total energy of compression of individual tableting materials do not substantially differ for the mixing period of 5 min at a frequency of 17 rev/min and a period of 2.5 min at the double frequency of 34 rev/min. The values of this energy, under these conditions of mixing with a lubricant, are the highest in Prosolv SMCC 90 and the mixture of Avicel PH-102 and Aerosil 255. A mixing time of 2.5 min and a frequency of 17 rev/min increase the value of the total energy for the mixture of Avicel PH-102 and Aerosil 200. The given course of the dependence of total energy again corresponds to the component of the total energy the energy for friction E_1. In the values of the energy accumulated by the tablet after compression E_2 there are again no marked differences for the twofold time and frequency of mixing and the highest values are recorded in Prosolv SMCC 90 and a mixture of Avicel PH-102 and Aerosil 255. The highest values of the energy of decompression E_3 are recorded in Prosolv SMCC 90 for all mixing times. In general, nevertheless, at this energy there are no marked differences in the values from the standpoint of the

conditions of mixing. Plasticity is decreased with an increase in the time or intensity of mixing in Avicel PH-102 and in a mixture of Avicel PH-102 and Aerosil 200, in the two remaining mixtures it is even for all conditions of mixing.

Tensile strength and disintegration time of tablets

Figure 3 shows the strength of tablets for varying conditions of mixing with magnesium stearate. The graph shows a slight decrease in strength due to doubling the time or frequency of mixing, where there are no statistically significant differences between the values. It indicates at a more perfect formation of the film of the lubricant on the carrier substance. The highest values of strength are shown by Prosolv SMCC 90, the values of other mixtures are similar, excepting the tablets from Avicel PH-102 and Aerosil 200 which show slightly lower values of strength. If we compare the result of the strength of tablets with and without magnesium stearate (Fig. 1), then it is clear that the addition of magnesium stearate does not markedly decrease tablet strength in Prosolv SMCC 90 and the mixtures of Avicel PH-102 with both types of Aerosil, but it decreases it markedly in Avicel PH-102, which again demonstrates the existence of competi-

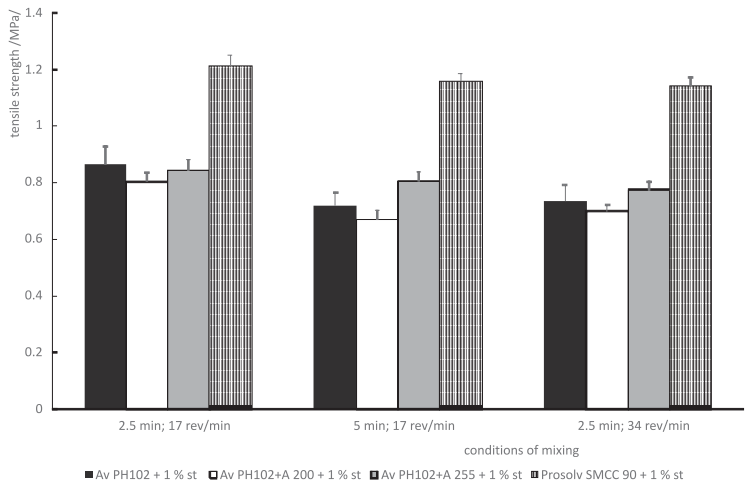

Figure 3. Tensile strength of tablets at the compression force of 3.5 kN: tableting materials with magnesium stearate (different conditions of mixing)

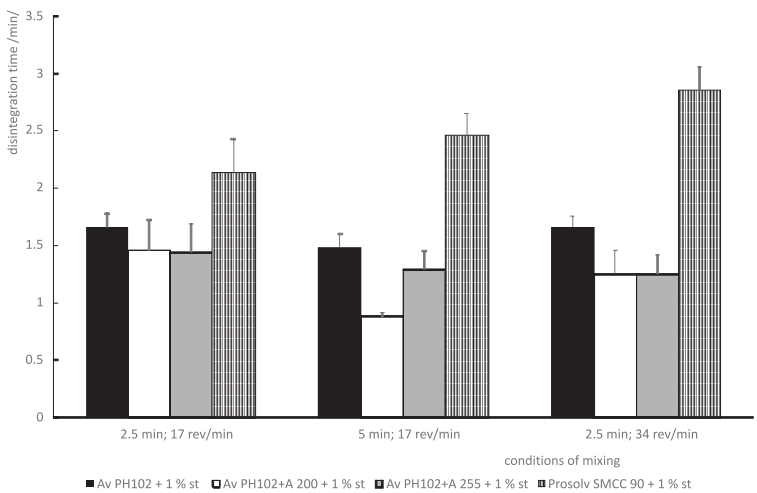

Figure 4. Disintegration time of tablets at the compression force of 3.5 kN: tableting materials with magnesium stearate (different conditions of mixing)

tive inhibition of the binding sites for magnesium stearate by colloidal silicon dioxide, both in the physical mixture and in the co-processed dry binder (7).

Disintegration time of tablets presented in Figure 4 is prolonged by doubling the time and frequency of mixing with the lubricant only in Prosolv SMCC 90, which would indicate a more perfect formation of the film of the lubricant. Its values are in this substance the highest, which corresponds to the highest strength of tablets. In the case of other tableting materials, the disintegration time is either not markedly changed (Avicel PH-102) or it is rather decreased (Avicel PH-102 with both types of Aerosil) due to changes in the conditions of mixing.

Testing the homogeneity of distribution of the lubricant in tablets

In Avicel PH-102 and Prosolv SMCC 90, the best homogeneity was found under the mixing conditions of 2.5 min 34 rev/min. In the case of other conditions of mixing, larger agglomerations of magnesium stearate were recorded. In the mixture of Avicel PH-102 and 2% Aerosil 200, the best homogeneity was for the condition of mixing 5 min and 17 rev/min, in the mixture of Avicel PH-102 and Aerosil 255, on the other hand, the best homogeneity was obtained under the conditions of mixing of 2.5 min 17 rev/min.

CONCLUSION

In conclusion, it can be stated that the total energy of compression increased with compression force in all tableting materials, the highest being in Prosolv SMCC 90. After addition of magnesium stearate its values did not differ under the conditions of mixing of 5 min 17 rev/min and 2.5 min 34 rev/min. Plasticity slightly decreased with compression force and in mixtures with magnesium stearate it was not influenced by the conditions of mixing. Tablets from Prosolv SMCC 90 and Avicel PH-102 showed a markedly higher strength than the tablets from the mixtures of Avicel PH-102 and both types of Aerosil. The addition of magnesium stearate markedly decreased the strength of tablets from Avicel PH-102. Increased time and frequency of mixing resulted in a further decrease in strength. Disintegration time was longer in tablets from Avicel PH-102 and Prosolv SMCC 90, due to the effect of adding magnesium stearate. In tablets from Prosolv SMCC 90, disintegration time was prolonged also by the influence of a longer time and higher frequency of mixing with the lubricant. Different conditions of mixing did not influence homogeneity of magnesium stearate uniformly.

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RELATIVE BIOAVAILABILITY OF RISEDRONATE SODIUM ADMINISTERED IN SUPERABSORBENT COPOLYMER PARTICLES *VERSUS* ORAL SOLUTION TO NORMAL HEALTHY RABBITS

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Abstract: In this study, sustained release superabsorbent copolymer particles have been prepared and analyzed to increase bioavailability of orally administered risedronate sodium. Formulations were prepared by free radical polymerization of combination of 2-hydroxyethyl methacrylate (HEMA), itaconic acid (IA), polyvinyl pyrrolidone (PVP) / chitosan (CTS) by using ethylene glycol dimethacrylate (EGDMA) as crosslinker, potassium persulfate as initiator, and N,N,N,N-tetramethylethylene diamine as activator. Formulations were successfully loaded with risedronate sodium. Formulations as gel particles encapsulated in hard gelatin were analyzed to estimate drug content. The maximum plasma drug concentration (C_{max}) and its corresponding time (T_{max}), area under the curve and relative bioavailability (with reference to oral solution of drug administered) were calculated. It was found a marked increase in T_{max} with lower C_{max} that confirmed the multiparticulte system to deliver drug at controlled rate. The results of relative bioavailability after oral administration of these formulations indicated a remarkable increase in the bioavailability.

Keywords: 2-hydroxyethyl methacrylate, itaconic acid, chitosan, risedronate sodium, superabsorbent polymer, relative bioavailability

A multiparticulate delivery system can provide orally administered drug a substantial area of GI tract for drug delivery at novel rate. Multiparticulate systems developed for novel drug release are generally comprises of granules, pellets or microparticles. The active therapeutic agent is present in the several small units that can be set in a capsule or formulated as a tablet for the proper delivery of drug. Because of their specific features, the subunits of multiparticulate system are less affected by time of gastric emptying and other GI tract variations. Hence, they smoothly pass GI tract and cause less irritation (1). Moreover, they provide better bioavailability and less chance of toxicity (2). After the oral administration these particles pass the GI tract at constant rate. Generally, the diameter of individual particle is less than 2 mm, so it can leave the stomach at constant rate even the pyloric sphincter is closed. This movement of particles at the constant rate results in a batter bioavailability of drug with less variation in its plasma levels. Multiparticulate drug delivery systems are safer than single unit novel formulations. For example, novel monolithic tablet can deliver ultra high contents of drug if its covering accidently rupture and can results in systemic toxicity. However, in these systems, the therapeutic substance is placed in several independent units and any damage to single unit deliver the sudden release of drug only from that. This characteristics reduces the safety issues related to novel formulations (3).

Various polymers are being utilized to prepare orally administered multiparticulate system. Superabsorbent copolymers have capability to absorb large volume of water, usually called hydrogels, that can be an option to prepare these systems (4). A hydrogel is comprised of a network of crosslinked polymer that swells in an aqueous environment without being dissolved in it. The liquid absorbed by polymer network prevents it from being compact mass (5). Hydrogel can be classified on the basis of the cross-linkages responsible of its appropriate three-dimensional structures. The forces responsible for appropriate networking might be physical or chemical in nature. Mostly, the polymer chains are joined by covalent bonds (6). Their

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porous structure can be modified by altering the density of inner cross-links and their affinity for aqueous medium. The porosity describes the drug loading capacity of hydrogel and subsequent release at controlled rate from network structure. This special feature of hydrogels makes them important for their use as drug delivery systems. In spite of several valuable features, hydrogels have some drawbacks. Their low tensile strength can affect drug holding capacity and might result in release of loaded drug before the appropriate time or specific site. The dispersion of drug in the polymer network is another problem, as commonly occurs when hydrophobic drug is attempted to load. The excessive swelling or formation of large pores in most of the hydrogels result in high release rate that directly affect drug concentration required at specific time. Gels administration is also problematic and requires proper formulation (7).

Many efforts have been made to utilize biodegradable polymers because of their compatibility with physiological system. Their features can be modified by slightly altering their structure (8). The swelling capacity of polymer describes the release rate of drug and by using various combinations of monomers/polymers copolymer of desired features can be developed. A multiparticulate system comprised of particles of super absorbent copolymer can

Table 1. Analysis of different pharmacokinetic parameters of risedronate sodium oral solution.

Rabbit	C_{max} (ng/mL)	T_{max} (h)	AUC_{last} (mg/mL × h)	AUC_{extra} (mg/mL × h)	AUC_{total} (mg/mL × h)
R1	8.99	0.47	0.027571	0.000473	0.028044
R2	8.64	0.51	0.024643	0.000259	0.024901
R3	8.86	0.53	0.025535	0.000246	0.025782
R4	9.01	0.49	0.027421	0.000643	0.028064
Mean	8.875	0.5	0.0262925	0.00040525	0.02669775
SD	0.170195965	0.025819889	0.001437907	0.000189618	0.001606854

Table 2. Analysis of different pharmacokinetic parameters of risedronate sodium formulation S.

Rabbit	C_{max} (ng/mL)	T_{max} (h)	AUC_{last} (mg/mL × h)	AUC_{extra} (mg/mL × h)	AUC_{total} (mg/mL × h)
R1	3.52	6.13	0.034772	0.0000185	0.034787
R2	3.56	6.27	0.0362275	0.000020424	0.036247
R3	2.74	5.89	0.026721	0.000021	0.026743
R4	2.87	5.76	0.030646	0.000015	0.030660
Mean	3.1725	6.0125	0.03209163	0.000018731	0.0321093
SD	0.42797001	0.230127	0.00429043	0.0000027072	0.0042891

Table 3. Analysis of different pharmacokinetic parameters of risedronate sodium formulation F.

Rabbit	C_{max} (ng/mL)	T_{max} (h)	AUC_{last} (mg/mL × h)	AUC_{extra} (mg/mL × h)	AUC_{total} (mg/mL × h)
R1	3.96	5.86	0.035182	0.000006	0.035188
R2	4.08	6.17	0.043958	0.00001	0.043968
R3	3.98	6.04	0.035143	0.00001	0.035153
R4	4.19	5.92	0.037193	0.000009	0.037202
Mean	4.0525	5.9975	0.037869	0.00000875	0.037878
SD	0.105633	0.137204	0.004170689	0.00000189297	0.004172

Table 4. Percent relative bioavailability of risedronate sodium formulation S.

Rabbit	AUC_{total} (mg/mL × h) of formulation S1	AUC_{total} (mg/mL × h) of oral solution	% relative bioavailability (F)
R1	0.034787	0.028044	124.044359
R2	0.036247	0.024901	145.564435
R3	0.026743	0.025782	103.727407
R4	0.030660	0.028064	109.250285
Mean	0.0321093	0.02669775	120.646621
SD	0.0042891	0.001606854	18.6956178

Table 5. % relative bioavailability of risedronate sodium formulation F.

Rabbit	AUC_{total} (mg/mL × h) of formulation (S)	AUC_{total} (mg/mL × h) of oral solution	% relative bioavailability (F)
R1	0.035188	0.02804	125.474255
R2	0.043968	0.0249	176.571222
R3	0.035153	0.02578	136.347064
R4	0.037202	0.02806	132.561288
Mean	0.037878	0.0267	142.738457
SD	0.004172	0.00161	23.0009662

be developed for appropriate delivery of drug to GI tract at controlled rate.

In present work, we have developed a multiparticulate drug delivery system comprised of super absorbent copolymer by internetworking of 2-hydroxyethyl methacrylate (HEMA) and itaconic acid (IA) with polyvinyl pyrrolidone (PVP)/chitosan (CTS). Gel particles were administered orally in hard capsules. Blood samples were collected and analyzed to estimate drug content. The maximum plasma drug concentration (C_{max}) and its corresponding time (T_{max}), area under the curve and relative bioavailability (with reference to oral solution of drug administered) were calculated. The results of relative bioavailability confirmed the remarkable increase in bioavailability of the risedronate sodium.

MATERIALS AND METHODS

Materials

Deionized highly purified water from a Barnstead Lab Tower EDI type 1 water purification system from Thermo Fisher Scientific (Laboratory Products Group) Waltham, United States was used in all procedures. 2-Hydroxyethyl methacrylate (HEMA) 97%, itaconic acid (IA) 96%, polyvinyl pyrrolidone (PVP) having average molecular weight 40,000, chitosan (CTS) 93% (w/w) having molecular weight 60,000-120,000, ethylene glycol dimethacrylate (EGDMA) 98%, potassium persulfate (KPS) 99.99%, N,N,N,N-tetramethylethylene diamine (TEMED) 99%, tetrabutylammonium bromide (TBAB) 98%, sodium phosphate 96%, etidronate disodium hydrate ≥ 97%, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-2Na) 98.5% and risedronate sodium 97% were purchased from Sigma-Aldrich, Germany. Rabbits (2.0-2.5 kg) were purchased from local market. All solvents used were of analytical grade.

METHODS

Preparation of HEMA-CO-IA/PVP copolymer particles

The method of solution polymerization was utilized after modification for the preparation of copolymer as reported by Li et al. (9). The weighed quantities of HEMA (9 g/100 g of solution), IA (2 g/100 g of solution) and PVP (1 g/100 g of solution) were dissolved in water/ethanol mixture while stir-

ring. EGDMA (0.7 g/100 g of solution) as crosslinker, potassium persulfate (0.1 g/100 g of solution) as initiator and N,N,N,N-tetramethylethylene diamine (0.6 g/100 g of solution) as activator were utilized. After appropriate mixing, the resultant clear solution was introduced into glass tubes. The solution, after bubbling with nitrogen, was subjected to heat at 50°C for 24 h. After this time, the tubes were cooled at room temperature and copolymer in cylindrical forms was collected, washed with ethanol/water mixture for the removal of unreacted materials (10), dried and fractionated with sieves to form superabsorbent copolymers particles. These superabsorbent copolymer particles of HEMA-CO-IA/PVP were designated as formulation S.

Preparation of HEMA-CO-IA/CTS copolymer particles

For the preparation of co-polymer, weighed amounts of HEMA (9 g/100 g of solution), IA (2 g/100 g of solution) and CTS (1 g/100 g of solution) were utilized. EGDMA (0.7 g/100 g of solution) as crosslinker, potassium persulfate (0.1 g/100 g of solution) as initiator and N,N,N,N-tetramethylethylene diamine (0.6 g/100 g of solution) as activator were used. CTS along IA was dissolved in distilled water at room temperature by continuous stirring in a flask equipped with magnetic stirrer and thermometer. After appropriate dissolution, the resultant solution was heated up to 70°C, weighed amount of HEMA was added with subsequent addition of EGDMA, potassium persulfate and N,N,N,N-tetramethylethylene diamine. After proper mixing, the resultant solution was introduced into glass tubes. These tubes were subjected to heat at 50°C for 12 h after bubbling with nitrogen. After the specific time period these tubes were cooled and copolymer in cylindrical form was collected, washed with ethanol/water solution for removal of unreacted substances (10), dried and fractionated with sieves to form superabsorbent copolymers particles. These superabsorbent copolymer particles of HEMA-CO-IA/CTS were designated as formulation F.

Loading of active substance into crosslinked copolymer

The loading of drugs was achieved by soaking the SAP particles for a week in drug solution. A 1% w/v drug (risedronate sodium) solution in ethanol–water mixture was found to be sufficient for

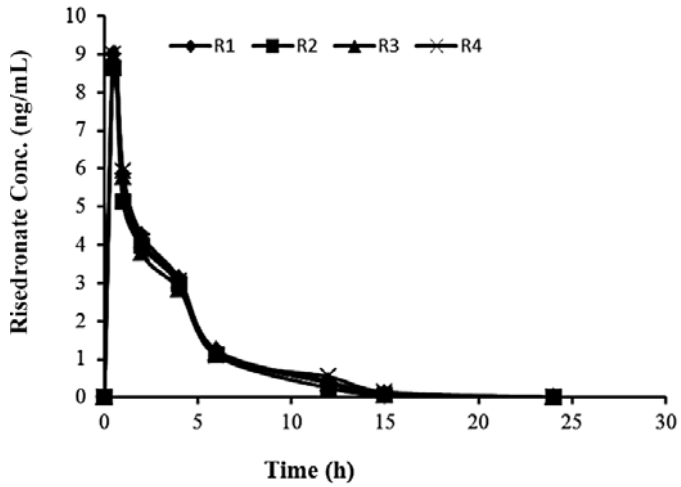

Figure 1. Plasma drug concentration (ng/mL) of risedronate sodium of oral solution

Table 6. 2-Sided Dunnett test analysis of oral solution against formulation S and formulation F with standard deviation; p value shows significant difference.

Parameters	Oral solution	Formulation S	Formulation F	p value
AUC_{total} (mg/mL × h)	0.026 ± 0.001	0.0321 ± 0.004	0.379 ± 0.004	0.006
C_{max} (ng/mL)	8.875 ± 0.170	3.172 ± 0.427	4.052 ± 0.105	0.000
T_{max} (h)	0.500 ± 0.025	6.012 ± 0.230	5.997 ± 0.137	0.000

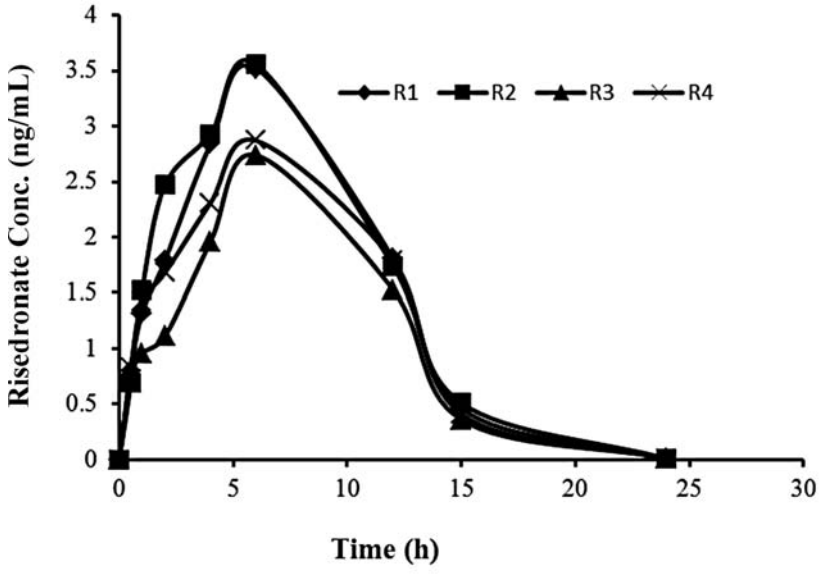

Figure 2. Plasma drug concentration (ng/mL) of risedronate sodium of formulation S

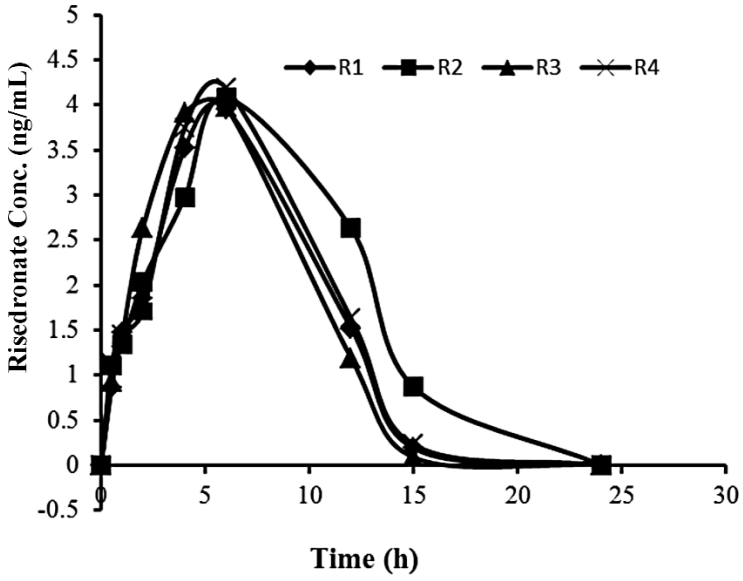

Figure 3. Plasma drug concentration (ng/mL) of risedronate sodium of formulation F

this purpose. After achieving the equilibrium, SAP particles were dried to remove solvent. Percentages of loaded drug were determined by extraction or swelling method (11).

Study design

The study utilized a balanced, single dose, parallel design (12). Twelve rabbits of 2.0 to 2.5 kg of weight participated in the study. The rabbits were assigned randomly to one of three groups receiving: drug oral solution, formulation S in capsule form and formulation F in capsule form.

Dosing and pharmacokinetic sampling

Rabbits of 2.0 to 2.5 kg of weight overnight fasted were utilized (only water was provided). The oral solution having required dose of drug and weighed amount of SAP particles according to

required dose (0.6 mg per kg of rabbit weight) was administered orally in hard capsules. Blood samples (1 mL) were collected from ear marginal vein immediately after dosing and at 0.5, 1, 2, 4, 6, 12, 15 and 24 h. Only water was provided to rabbits during the trial (13). Risedronate sodium was extracted and analyzed by using a validated HPLC method as stated below.

Analytical method

Risedronate sodium stock solution was prepared at a concentration of 1 mg/mL in de-ionized water. Different working standards at concentration range from 1 to 10 µg/mL of risedronate sodium were prepared from stock solutions by serial dilutions with deionized water.

Blood from rabbit was collected in EDTA-2Na (1.5%, w/v) tubes, centrifuged at 4000 rpm for 15 min and upper layer (plasma) was collected. Plasma drug standards were prepared by incorporating risedronate sodium working standards in plasma at concentrations ranging from 10 to 100 ng/mL to construct standard/calibration curve for quantitative analysis. One hundred fifty (150) µL of each plasma drug standard was diluted with 1.4 mL of pure water, deproteinized by the addition of 450 µL of 10% (w/v) trichloroacetic acid (TCA) and centrifuged for 15 min (at 1500 rpm). The supernatant

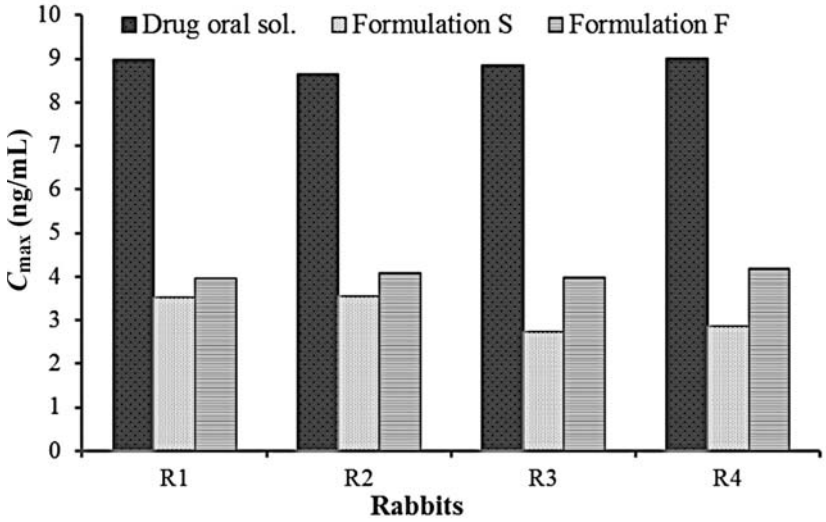

Figure 4. C_{max} of risedronate sodium of oral solution and formulations

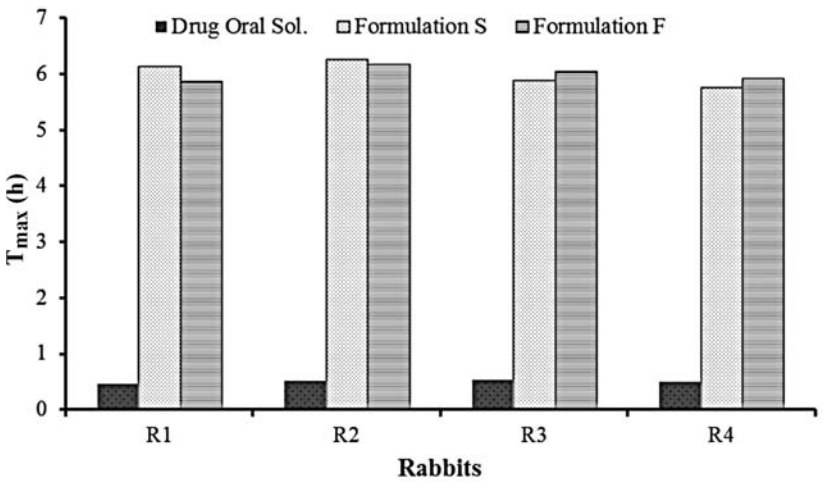

Figure 5. T_{max} of risedronate sodium of oral solution and formulations

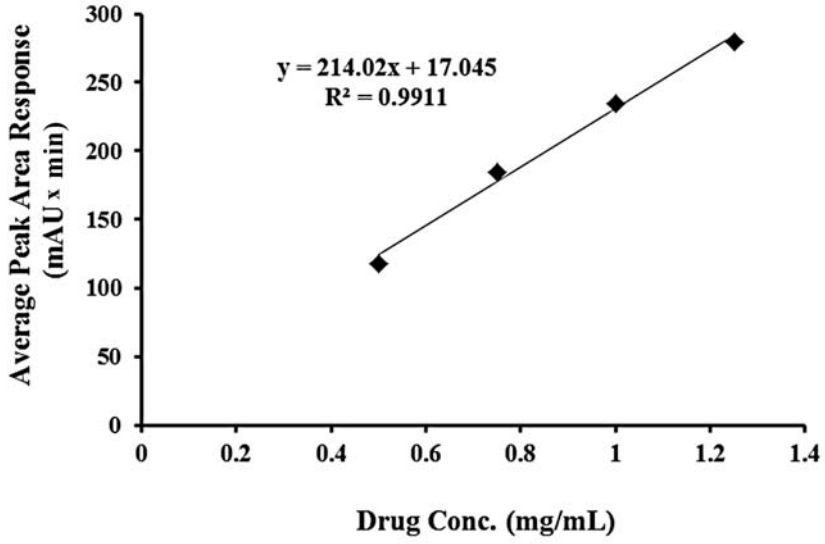

Figure 6. Average peak area response drawn against known drug concentration

was collected and 50 µL of $CaCl_2$ (1.25 M) along 57 µL of 30% (w/v) NaOH were incorporated to induce the precipitate formation. After proper mixing, each sample was centrifuged for 10 min (at 4500 rpm). The precipitate/pellet was collected and dissolved in 400 µL of HCl (1 M), 25 µL of $CaCl_2$ (1.25 M) and 90 µL of 30% NaOH. The critical step in extraction of risedronate sodium from plasma was when each sample was centrifuged further for 10 min (at 4500 rpm) and the pellet was dissolved in 50 µL of HCl (1 M) and 45 µL of 30% NaOH, and a white precipitate was formed. The sample was centrifuged for 10 min (at 4500 rpm) and this last precipitate formed was dissolved in 80 µL of 0.025 M EDTA-2Na and 920 µL of mobile phase was added, mixed, ?ltered and subjected to analysis by HPLC (14).

Analytical method conditions

The mobile phase for separation of risedronate in plasma consisted of buffer (5 mM TBAB ion-pair reagent, 11 mM sodium phosphate, 1 mM etidronate disodium hydrate and 1.5 mM EDTA-2Na) and methanol (88 : 12, v/v), adjusted to pH 6.75, and was pumped at a flow rate of 1.0 mL/min. The injection volume was 100 mL and the detection wavelength was 262 nm using reverse phase column (i.d. 5 µm, 4.6 × 250 mm, ODS-2). Peak areas were used for quantitative analyses.

***In vivo* data analysis**

Pharmacokinetic parameters were calculated employing Kinetica ver. 4.4 from the concentration time data for an individual drug. Maximum plasma drug concentration (C_{max}) and its corresponding time (T_{max}) were directly calculated from the plasma concentration-time curve. The trapezoidal rule was utilized to determine area under the curve (15). Relative bioavailability was calculated with reference to oral solution of drug administered (13):

$$\% \text{ relative bioavailability of test product (F)} = \frac{AUC_{0-\infty} \text{ test product}}{AUC_{0-\infty} \text{ oral solution}} \times 100$$

Statistical analysis

The data of pharmacokinetic parameters, such as AUC, C_{max} and T_{max} were analyzed using 2-sided Dunnett test by considering p value less than 0.05 as significant. For this purpose, SPSS version 13 program was used.

RESULTS AND DISCUSSION

Effect on C_{max}, T_{max} and AUC

Average values of C_{max}, T_{max} and AUC were observed to be 8.875 ± 0.170195965 ng/mL, 0.5 ± 0.025819 h and 0.02669775 ± 0.001606854 mg/mL × h, respectivrly, for oral drug solution, 3.1725 ± 0.42797001 ng/mL, 6.0125 ± 0.230127 h and 0.0321093 ± 0.0042891 mg/mL × h, respectively, for formulation S and 4.0525 ± 0.105633 ng/mL, 5.99 ± 0.137204 h and 0.037878 ± 0.004172 mg/mL × h, respectively, for formulation F. The values of C_{max}, T_{max} and AUC were shifted to new one when formulation results were compared to conventional dosage form i.e., oral solution of drug. The two

copolymer formulations showed a significantly greater T_{max} values than the drug oral solution, but showed a lower C_{max}. It was concluded that risedronate sodium administered in the form of oral solution has a rapid absorption that reflects the normal behavior of immediate release formulations. As the values of C_{max}, T_{max} and AUC of both of copolymer formulations were shifted to new one that behavior proved their sustained release behavior.

The application of 2-sided Dunnett test (Table 6) revealed that value of AUC is the lowest for the oral solution with values of 0.026 ± 0.001 and yielded higher values for formulation S and still further higher values for formulation F. This indicated that it was considerable increase in the existence of residronate in the plasma due to better control of polymeric system developed. Values of C_{max} showed that there was large reduction in the maximum drug concentration, favoring marked delay in the residence of drug in plasma with lower for formulation S than formulation F. T_{max} was found to be the highest for oral solution as compared to formulations S and F, indicating that there is marked reduction in the rapid absorption and elimination of drug from animal body, alternatively suggesting that drug remain available for action in the plasma for a longer period of time causing sustained effect of multiparticulate system. The highly significant values of p calculated from above data proved the better control of superabsorbent co-polymer particles over oral solution.

Effect on relative bioavailability

Relative bioavailability (%) was found to be 120.646621 ± 18.6956178 for formulation S and 142.738457 ± 23.0009662 for formulation F, respectively. The results indicate that the bioavailability of risedronate sodium was improved significantly when administered in the form of multiparticulate formulation. The results expose that the HEMA-co-IA/PVP and HEMA-co-IA/CTS copolymers can be used as drug carriers to improve bioavailability of risedronate sodium.

CONCLUSION

Multiparticulate systems comprised of HEMA-co-IA/PVP and HEMA-co-IA/CTS copolymer particles superabsorbent were successfully established and loaded with water soluble drugs model drug risedronate sodium. The bioavailability of risedronate sodium was greatly increased when administered in the form of multiparticulate drug delivery system. C_{max}, T_{max} and AUC were shifted to new values when the results were compared with oral solution. These results ensure that the HEMA-co-IA/PVP and HEMA-co-IA/CTS copolymers can be used in different formulation to enhance the bioavailability of risedronate sodium.

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FORMULATION AND EVALUATION OF MICROSPHERES CONTAINING LOSARTAN POTASSIUM BY SPRAY-DRYING TECHNIQUE

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Abstract: Despite numerous applications of microspheres, few works devoted to the preparation of microspheres containing cardiac medications have been published. This study presents the potential of receiving microspheres containing losartan potassium, based on a matrix containing Eudragit L30D55. The study focuses on the possibilities of controlled release of losartan potassium from microspheres in order to reduce the dosage frequency, and also provides information on the effect of the addition of excipients to the quality of the microspheres. Microspheres are monolithic, porous or smooth microparticles ranging from 1 to 500 microns in size. For the preparation of microspheres containing losartan potassium, the spray-drying method was used. The performed study confirmed that the spray-drying technology used to obtain microspheres meets the criteria of size and morphology of the microparticles. The assessment of the kinetics of losartan potassium release from the examined microspheres demonstrated that the release profile followed the first- and/or zero-order kinetics. The use of spray-drying techniques as well as Eudragit L30D55 polymer matrix to obtain the microspheres containing losartan potassium makes it possible to obtain a product with the required particle morphology and particle size ensuring the release of the active substance up to 12 h.

Keywords: losartan potassium, microspheres, spray drying, Eudragit L30D55, kinetics

Among the sustained release drug forms, microspheres are treated with great interest and hopes. Microspheres find more and more therapeutic use, mainly among cancer and ophthalmic patients, but also in the routine therapy of chronic diseases (1, 2). Microspheres are monolithic, porous (3) or smooth (2) microparticles ranging from 1 to 500 microns (3), or 1000 microns (1, 4) of the matrix characters (5). They are built from different types of polymers, in which the drug substance is incorporated (dissolved or suspended) (1, 3), rarely emulsified (6). In medicine, the microspheres with sizes ranging from 10 microns to 50 microns, injected subcutaneously (6), are used most commonly. In clinical trials, the following substances have been tested: 5-fluorouracil, aclarubicin, doxorubicin, methotrexate (confirming the high concentration of the drug in the affected tissue, and at the same time a low concentration in the systemic circulation) (4, 5). Attempts to obtain microspheres have also been taken by using acyclovir, methylprednisolon, gentamicin, insulin, calcitonin, furosemide, amoxicillin, vancomycin (7). The microspheres are used in the field of vaccines preparation (6), subcutaneous implants (5, 8), and also in ophthalmology (e.g., timolol maleate in the form of a mucoadhesive system, which prolongs the contact time of the active substance with an eyeball (2), pilocarpine nitrate in the form of albumin microspheres having prolonged release profile (9)). Microspheres find the significant use in hemoembolization of blood vessels and radioembolization (10). There are also attempts at the use of paclitaxel to inhibit the growth of lung cancer (10), and of tenofovir in the prevention of HIV (11). Test results also indicate the possibility of using microspheres in bioadhesive drug forms (4, 7, 12).

The substances used in the production of the microspheres must be biocompatible, have suitable

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mechanical properties, and have a predictable release profile (5). Polymer materials of natural or synthetic origin (1, 2, 5, 13) are used most frequently.

The advantages of microspheres include: biocompatibility, extended release profile and the possibility of incorporation into the polymer matrix of unstable substances, such as proteins or nucleic acids (1, 7, 14, 15). The microspheres are used for drugs characterized by low bioavailability after oral administration, or those which are inactivated in the gastrointestinal tract (15, 16).

Despite numerous applications of microspheres, only a few published works devoted to the preparation of microspheres containing cardiac medications have been published. This study presents the potential of receiving microspheres containing losartan potassium, based on a matrix containing Eudragit L30D55.

Losartan potassium, chosen for testing, is commercially available only in the form of one compartment tablets (a classic tablets at a dose of 25, 50, 100 mg of active substance) or combined with hydrochlorothiazide. Due to the bioavailability of losartan potassium at a level of about 33%, half-life in the human body of about 2 h, and very frequent use in a long-term therapy (e.g., in hypertension), there is a need to develop oral formulations of losartan that can guarantee sustained and controlled release of active substances that could simultaneously improve the bioavailability (17, 18). The administration of losartan potassium in the form of a sustained release should be characterized by reaching rapidly the concentration of therapeutic effect. Then, a properly chosen polymer matrix should maintain plasma concentration within the therapeutic range for longer time (about 8-12 h) (19).

The aim of this study was to develop a preparation with losartan potassium with a sustained release profile, and assess the usefulness of this form of losartan potassium in relation to a commercially available standard. All preparations were developed on the basis of microspheres (a multicompartment form).

EXPERIMENTAL

Materials

In this work, the following substances were used: losartan potassium (Valeant, ICN Polfa Rzeszów SA), triethyl citrate (Sigma-Aldrich Chemie GmbH), Eudragit L30D55 dispersion of 30% w/w, (Evonik, Degussa), 50 mg tablets of losartan potassium (trade standard).

METHODS

Technology for obtaining the microspheres

For the preparation of microspheres containing losartan potassium, the spray-drying method was used (20-23). Aqueous dispersion (30% w/w) of methacrylic acid - Eudragit L30D55 with losartan potassium and/or triethyl citrate (TEC) in an amount of 10% calculated on the dry matter of the reaction substrates suspended in 100 mL of purified water were subjected to drying. The suspension before and during the process of drying, was continuously mixed for 1 h using a magnetic stirrer. To carry out the spray drying process, a spray dryer Büchi Mini Spray Dryer B-191 was used. The composition of each dried mixtures are shown in Table 1.

The spray-drying process was carried out using the following parameters of spray drying: a peristaltic pump output 10%, inlet temperature 150^{o}C, the aspirator capacity 80%. Spray drying was carried out by using a nozzle with a diameter of 0.7 mm at the air flow rate of 600 L/h and pressure of 4 bars.

Table 1. Quantitative composition of the substances used in the various formulations (24).

The ratio drug : polymer	Amount of losartan potassium [mg]	Water volume [mL]	Amount of dispersion of Eudragit L30D55 [mg]	Dry matter Eudragit L30D55 [mg]	Volume of triethyl citrate (TEC) [µL]	Sample code
1 : 1	200.00	100	666.67	200.00	35.09	1 : 1 TEC
1 : 2	200.00	100	1333.33	400.00	52.63	1 : 2 TEC
1 : 3	200.00	100	2000.00	600.00	70.18	1 : 3 TEC
1 : 4	200.00	100	2666.67	800.00	87.72	1 : 4 TEC
1 : 5	200.00	100	3333.33	1000.00	105.26	1 : 5 TEC
1 : 1	200.00	100	666.67	200.00	---	1 : 1
1 : 3	200.00	100	2000.00	600.00	---	1 : 3

Received microspheres were collected in a collector (24).

Scanning electron microscopy (SEM)

Observation of the microstructure of samples was performed by using a high resolution scanning electron microscope (SEM) SUPRA25 Carl Zeiss Co., equipped with a X-ray spectrometer with energy dispersion from EDAX. Viewed samples of analyzed materials were imaged without the application of conductive layers. Photos of the microspheres structure were observed at magnifications of 1000×, 5000× and 10000×, and the measurement of particle size at a magnification of 10000×. The size of particles was evaluated by using software for the image analysis provided with SEM.

Measurement of particles size

The research was carried out with Analysette 22 MicroTec of Fritsch Company using laser diffraction. The device is equipped with two solid-state lasers: green and infrared. The measuring range is from 0.08 to 2000 microns. The examined microspheres were ultrasonically dispersed in distilled water for 5 s before and during the measurement. Three tests of each powder were performed. The computational model of Fraunhofer Institute was used.

Evaluation of the effectiveness of losartan potassium coating (17)

The effectiveness of coating the losartan potassium by Eudragit L30D55 was calculated as the ratio of the actual to theoretical drug content in the obtained microspheres and expressed in percentage. The dependence is shown by the formula: EP = (Zr / Zt) × 100%, where: EP - coating efficiency, Zr - the actual content of losartan potassium in the studied microspheres, Zt - theoretical content of losartan potassium in the studied microspheres.

Study of the release of losartan potassium from the microspheres

The study was conducted according to the guidelines of Polish Pharmacopeia IX and US Pharmacopeia. Losartan potassium release from the microspheres was performed in a flow apparatus ERWEKA FRL800 + SE at 37 ± 1^{o}C. The flow rate of acceptor fluids was set at 4 mL/min. There were used two acceptor fluids: artificial gastric juice, pH ~1.2, and an artificial intestinal juice with a pH ~ 6.8. The release in artificial gastric juice (pH ~1.2) was carried out for a period of 2 h, then the acceptor liquid was changed to a buffer with a pH of ~6.8, and the test was continued for 6 h. Samples were taken as follows: at 15, 30, 60 and 120 min from artificial gastric juice and at 15, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 min after changing to artificial intestinal juice. The absorbance of the collected samples was measured using a UV-VIS spectrophotometer at a wavelength of 254 nm, using suitable acceptor fluid as reference. Losartan potassium concentration was calculated based on a standard curve with the equation:

$$y = 0.029x + 0.003, R^2 = 0.997.$$

Statistical analysis

The results of release losartan potassium from microspheres were statistically analyzed using Statistica 10 and Microsoft Office Excel 2007. The evaluation of the normality of the variables were tested by the Shapiro-Wilk test. The results of the analysis of the release profiles of microspheres were assessed using ANOVA analysis. The homogeneity of variance was tested by the Brown-Forsythe test. The significance of differences "each to each" for the average levels of release was tested in a *post-hoc* LSD (*Least Significant Difference*) test.

Analysis of Raman spectra

To clarify the phenomenon of release of losartan potassium in an artificial gastric juice (pH 1.2) in the presence of Eudragit L30D55, which has a release according to manufacture's product information at pH > 5.5, the analysis of Raman spectra was carried out. Studies on degree of deformation of microspheres structure containing losartan potassium were made using the Via Reflex Raman Spectrometer of Renishaw Co. equipped with a confocal microscope (Leica) with the possibility of samples observation in reflected and transmitted light. Excitation was carried out by using the beam of λ = 514 nm of argon-ion laser with a power of 50 mW. The adjustment range of the laser power was from 0.00005% to 100%. Measurements were recorded using a lens with a magnification of 50× and a long-distance lens (called: Long Working Distance - LWD) with a magnification of 20×. Raman spectra were recorded from 50 to 4100 cm^{-1}. The following microspheres were examined: 1 : 1 TEC 1 : 3 TEC 1 : 5 TEC, 1 : 1, and patterns of losartan potassium and Eudragit L30D55.

RESULTS

The spray-drying process was carried out in accordance with the specified methodology, and the resulting products were collected, weighed and their

size and morphology was determined by scanning electron microscopy techniques. Detailed results are shown in Table 2. Studies have shown that increasing the amount of the used polymer affects negatively the quality of the obtained microspheres: particle size increased but the morphology worsened. It could be observed that with an increasing amount of polymer, domination of oval forms and forms of irregular shape appeared, whereas spherical forms disappeared. The disproportion in the size of parti-

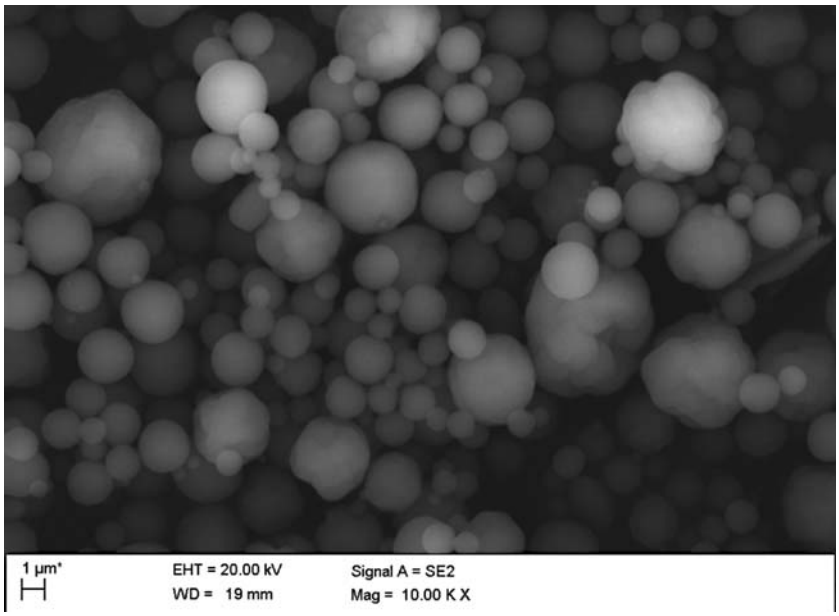

Figure 1. Sample 1 : 1 TEC - Scanning electron micrograph (SEM) of the microspheres containing losartan potassium and Eudragit L30D55 in ratio 1 : 1 with the addition of plasticizer: 10% triethyl citrate (magnification 10000×) (24)

Table 2. Characteristics of the spray dried microspheres of losartan potassium with Eudragit L30D55 in ratios from 1 : 1 to 1 : 5 with the addition of triethyl citrate (TEC) in an amount of 10% based on the dry matter of drug : polymer, and microspheres in the ratio of 1 : 1 and 1 : 3 which did not contain triethyl citrate (24).

Symbol of the sample	Product mass [mg]	Product efficiency [%]	Particle size [µm]	Morphology
1 : 1 TEC	172	43.00	0.82-6.97	Clearly spherical particles, very few forms of an oval shape
1 : 2 TEC	292	48.67	0.98-7.04	Spherical particles, some have a recess in the central part of the coat, a very few particles of irregular shape
1 : 3 TEC	415	51.88	1.06-7.71	Spherical particles, some have a recess in the central part of the coat, there are particles of irregular shape and parti cles having a recess in a portion of the coating
1 : 4 TEC	523	52.30	1.26-10.12	The presence of spherical particles with the recesses in th part of the coat, there are increasing number of particles of irregular shape, the current cone-like shape of the particles
1 : 5 TEC	650	54.17	1.24-10.45	Particles with a shape similar to a spherical portion having a recess in the shell, the presence of numerous particles of irregular shape
1 : 1	165	41.25	1.27-7.24	Mostly spherical form and forms a shape similar to a sphere with a slightly blurred edges, a few forms with different shape
1 : 3	354	44.25	1.95-12.96	Clearly visible forms of a very irregular shape, the absence of spherical form, a very large disparity in the size of particles

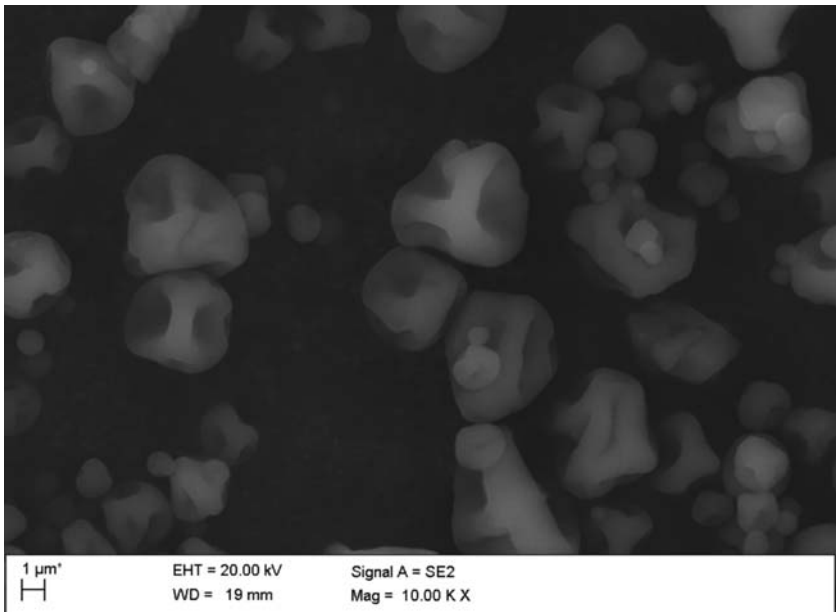

Figure 2. Sample 1 : 3 TEC - Scanning electron micrograph (SEM) of the microspheres containing losartan potassium and Eudragit L30D55 in ratio 1 : 3 with the addition of plasticizer: 10% triethyl citrate (magnification 10000×) (24)

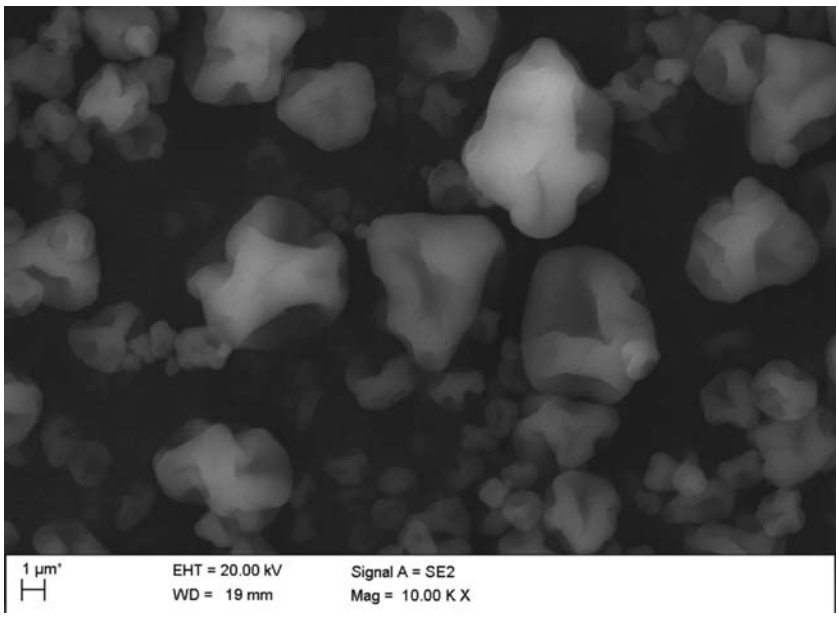

Figure 3. Sample 1 : 5 TEC - Scanning electron micrograph (SEM) of the microspheres containing losartan potassium and Eudragit L30D55 in ratio 1 : 5 with the addition of plasticizer: 10% triethyl citrate (magnification 10000×)

cles has increased**.** In addition to small particles of about 2 mm, particles bigger than 12 mm began to occur, which was not observed for dried microspheres containing a plasticizer (triethyl citrate). Comparing the dried microspheres with or without addition of triethyl citrate, its beneficial effect on the microspheres size can be stated; there was observed a small reduction in the average particle size and the quality of the microspheres improved. In the microscopic image, we can see spheroids which are much more homogeneous, morphologically correct and consistent with the literature standard, and a small

amount of structures with parameters deviating from the standard literature (1, 3, 4). Triethyl citrate also slightly improves the efficiency of the process in trials with the addition of plasticizer (by ca. 5%).

Figures 1-5 show the microspheres obtained by a spray-drying method at magnification of 10000× taken with an electron microscope. Figures 1-3 present the microspheres containing additive triethyl citrate in the matrix, while Figures 4 and 5 show the microspheres, which are composed of a polymer matrix which does not contain a plasticizer.

The size of microspheres, and scatter of their size can be a parameter characterizing the quality of the obtained microspheres and repeatability of the

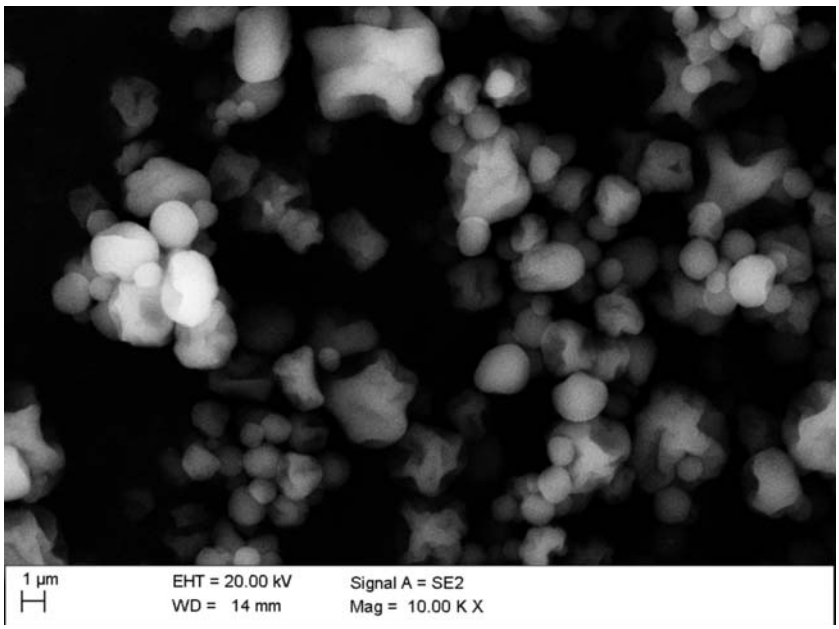

Figure 4. Sample 1 : 1 - Scanning electron micrograph (SEM) of the microspheres containing losartan potassium : Eudragit L30D55 1 : 1 without the addition of a plasticizer (magnification 10000×).

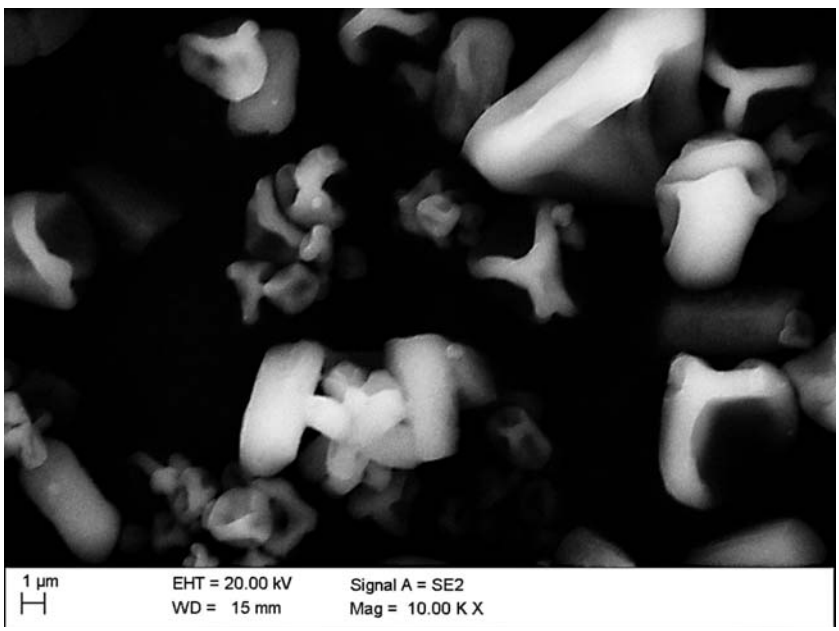

Figure 5. Sample 1 : 3 - Scanning electron micrograph (SEM) microspheres containing losartan potassium : Eudragit L30D55 1 : 3 without the addition of plasticizer (magnification 10000×) (24)

Table 3. Particle size distribution obtained by spray-dried technique.

Symbol of the sample	Quantile Q(x) [%]	Size [µm]	Density of dispersion CV [%]
1 : 1 TEC	10 50 90	1.253 3.592 8.363	3.91 6.23 5.93
1 : 2 TEC	10 50 90	1.562 4.195 9.106	4.18 7.91 9.74
1 : 3 TEC	10 50 90	1.674 4.831 12.062	5.37 5.66 4.77
1 : 4 TEC	10 50 90	1.663 5.026 11.784	3.01 4.18 4.47
1 : 5 TEC	10 50 90	2.122 6.441 14.165	0.85 0.64 1.19
1 : 1	10 50 90	0.929 4.415 11.826	1.47 0.47 1.05
1 : 3	10 50 90	1.588 5.579 13.321	3.47 5.01 3.85

Table 4. The efficiency of encapsulation with the percentage of losartan potassium spray dried microspheres.

Number of sample	Yield of microspheres formation [%]	Drug content in the microspheres [%]*	The efficiency of encapsulation [%]**
1 : 1 TEC	43.00	47.60	92.24
1 : 2 TEC	48.67	27.53	88.19
1 : 3 TEC	51.88	30.92	113.39
1 : 4 TEC	52.30	18.34	83.34
1 : 5 TEC	54.17	14.50	88.19
1 : 1	41.25	41.36	82.72
1 : 3	44.25	32.36	129.68

* The drug content in the microspheres expressed as the amount of experimentally released losartan potassium based on the matter of the microspheres released.
** Efficiency of encapsulation, expressed as the ratio of losartan potassium experimentally released relative to the amount of losartan potassium, calculated theoretically

process. The scatter and the average particle size for each sample are summarized in Table 3.

All the obtained samples show the normal distribution characteristics. With the increase in thickness of the polymer matrix, the size of the microspheres increases. The addition of the plasticizer reduces the particle size and improves the spreading density, thus, the particles become more homogeneous. All microspheres, in term of particle size, meet the criteria, but only the microspheres identified by symbols 1 : 1 TEC 1 : 2 TEC 1 : 3 TEC and 1 : 1 meet the morphologic criteria, which are sphericity and uniformity, and the absence of pits in the shell.

When examining encapsulation effectiveness for losartan particles, we calculated theoretical losartan potassium content in the microspheres, including the effectiveness of the process. Next, the content was compared to experimental content obtained by total release of losartan potassium from

the studied microspheres. The content of losartan in the individual microspheres was expressed as a percentage. The results are summarized in Table 4.

The ability of losartan potassium molecules encapsulation by Eudragit L30D55 is similar in all cases - it confirms the reproducibility of the spray drying process and guarantees obtaining virtually homogeneous particles with this method. Samples 1 : 3 TEC and 1 : 3 are interesting, as encapsulation effectiveness is here significantly higher, exceeding even 100% of the declared content calculated theoretically. Drug content in both cases is similar and reaches 30%. Sample 1 : 1 TEC exhibits the highest of drug content in the microspheres, and is characterized by high efficiency encapsulation and appropriate morphology confirmed by SEM studies.

Microspheres release studies were carried out for 8 h, or up to 100% release of drug content in the microspheres. The percentage of the released substance is shown in relation to the theoretical content present in the microspheres (Fig. 6).

Compared to the standard, the effect of prolonged action is shown by formulation 1 : 1 TEC. The release profile of this formulation is similar to the ZOK form (zero-order kinetics) (R^2 = 0.994,

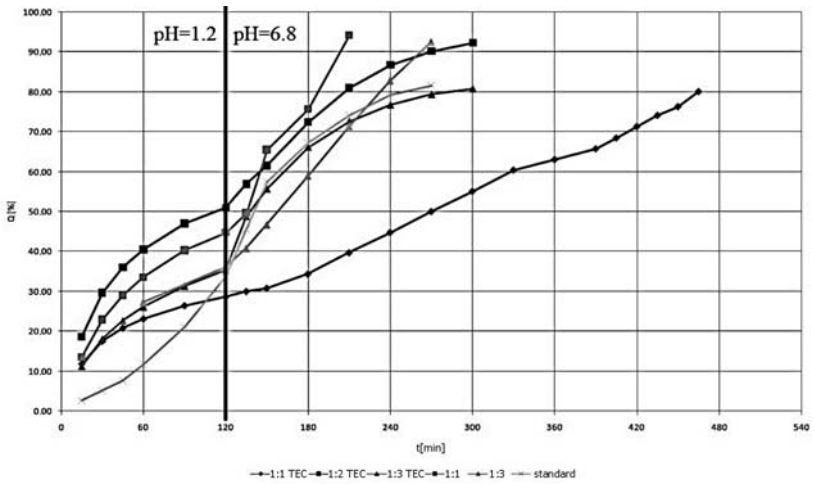

Figure 6. The release curves of the investigated microspheres losartan and standard preparation

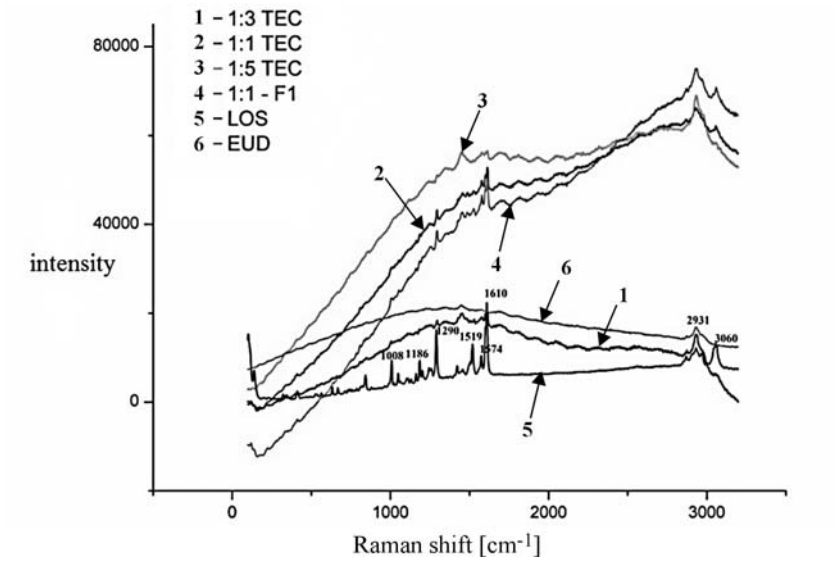

Figure 7. Raman spectra of the active substance (losartan potassium - LOD) and matrix polymer (Eudragit L30D55- EUD), and the selected microspheres

y = 0.142x + 11.78). The release time of the active substance compared to the standard is extended and the release profile is almost linear. However, none of the tested formulations show statistically significant differences from the standard at pH = 1.2, while the change of the acceptor fluid to artificial intestinal juice at pH = 6.8 results in differences shown only by two formulations 1 : 1 TEC and 1 : 1. This option 1 : 1 TEC allows to release the drug over a period of 8 h. The observation confirms the analysis of the release curves shown in Figure 6. The addition of the plasticizer also leads to a statistically significant change in release profiles of losartan potassium from the microspheres. The release time of losartan potassium after adding a plasticizer is extended. There are statistical differences between the formulations of microspheres 1 : 1 TEC and 1 : 1 as well as between 1 : 3 TEC and 1 : 3. There might be a relation between sphericity of microspheres and their release profile. Based on the analysis of release curves and scanning electron microscope images of microspheres, it can be assumed that more spherical microspheres release particles more slowly than their porous counterparts. There was no relation between the release profile of losartan potassium from the microspheres and the amount of the polymer matrix.

As far as release profiles are concerned, it is worth observing that at pH = 1.2, losartan potassium is released into an acidic medium. The attempt to explain this phenomenon, based on analysis of the Raman spectra (Fig. 7), was made. The analysis showed peak shifts in the structure of the microspheres for losartan potassium and Eudragit L30D55. We observed four shifts: three of them in the functional groups (3700-1500 cm^{-1}) and one outside the functional groups (1300-800 cm^{-1}). The following values of Raman effect are changed: from 1610 to 1613 cm^{-1}, from 1574 to 1578 cm^{-1}, from 1519 to 1527 cm^{-1} and from 1289 to 1295 cm^{-1}. In the case of losartan potassium, electron binding forces substitutions may involve the aromatic ring or the imidazole ring in the case of Eudragit, and we can observe a possibility of deformation of the C=O bindings of carboxyl group (one free and one substituent esterified ether). In all analyzed compounds, there are peaks at the wavelength of Raman effect, i.e. 2931 cm^{-1} and 3060 cm^{-1}, which confirm the presence of aliphatic groups and alkanes chains. In the range of 3000 to 1600 cm^{-1}, higher intensity peaks for Eudragit L30D55 are observed. This indicates the activation of vibration in polymer bonds in microspheres. In the range of 1800 to 1600 cm^{-1}, mainly double bonds, i.e. C=O and C=N, vibrate, whereas in the range 2000-1800 cm^{-1}, we can observe slight deformative vibrations of CH bonds in the aromatic rings, while, we can see the absorption of -COOH groups or NH amine salt in the range of 2700-2000 cm^{-1}. We can also observe that, in the range of 1200-500 cm^{-1}, the intensity of some peaks is reduced, which is visible in the Raman spectra of losartan potassium, and less prominent in the spectra of the molecules of the microspheres, and it is evident for the two peak values of Raman effect of 1008 cm^{-1} and 1186 cm^{-1}. This may indicate extinguishing part of vibration in the losartan molecule mainly between the bonds CC, CN and CO that vibrate in the range of 1300-500 cm^{-1}, it may be due to surrounding losartan particles by Eudragit particles.

The observed effect in the Raman spectra in Eudragit L30D55 molecule after mixing with losartan potassium may, to some extent, explain the observed, during the drug release from the microspheres, change in the properties of Eudragit L30D55 that according to datasheet, should be dissolved and released only at pH ≈ 5.5, while in our research, as a result of electron changes it could change its properties and release an active substance at pH = 1.2.

DISCUSSION

The concept of a modern dosage form is a targeted and controlled drug release, with prolonged activity, which, on the one hand, improves patient's comfort by reducing the dosing frequency, and on the other hand, ensures the absence of excessive changes in drug plasma level (25). The microspheres with a medicinal substance incorporated into a polymer matrix are forms that meet the above criteria (6).

The effect of the influence of plasticizer addition on the size and morphology of the obtained microspheres was analyzed in this study. Research of Rujivipat and Bodmeier (26) shown that the matrix containing in its composition copolymers of methacrylic acid is too rigid and requires the presence of plasticizer in order to improve its properties. It has been shown that the addition of plasticizer affects the efficiency of the spray-drying process and the size of the microspheres. The addition of triethyl citrate improves the efficiency of the process (efficiency gains of 5%) and also contributes to increasing regularity, sphericity and reduces the size of microspheres (1.92-8.36 μm). According to Sawicki and Makulec (27), triethyl citrate improves flexibility and adhesive properties of the polymer matrix, and has a beneficial effect on the morphology of obtained microspheres, while Snejdrova and

Dittrich (28) argue that the plasticizer can be used even in the quantity of 30%.

We also evaluated the effect of Eudragit L30D55 content on particle morphology and efficiency of the spray-drying process. During the study, it was observed that an increase in Eudragit L30D55 content was accompanied by increased efficiency of the process, however the quality of the obtained particles worsened. Different results were obtained by Irom et al. (17), who studied the morphology of chitosan microspheres with losartan potassium obtained by emulsifying and evaporation of the solvent. Using SEM method, they found that the quality of the microspheres improves with an increase of polymer content relative to active substance content.

In this study, the influence of the polymer content on the quality of the microspheres was clearly observed. We dried formulations containing losartan potassium and Eudragit L30D55 in 1 : 1 to 1 : 5 ratios. To each formulation, triethyl citrate was added as a plasticizer. It was found that increased amount of the polymer increases the particle size (Q10% quanta changing from 1.25 to 2.04 μm while Q90% of 8.36 to 12.55 μm). Morphology worsens proportionally to the polymer content – in the microscopic image, we can observe a reduced number of spherical forms and an increased number of forms of irregular shape. What is more, the size of microspheres also increased.

In the study of Esposito et al. (29) the effect of polymer concentration on the morphology of the obtained microspheres was evaluated, and it was shown that with the increasing amount of the polymer, microspheres size was also increased and their morphology was changed to a less spherical. In SEM photography, microscopic agglomerated particles could be detected. The size of the microspheres was increased due to an increase in polymer concentration. Rizi et al. (30) state in their study that a greater amount of solids present in the drying process leads to morphological abnormalities. Obtained microspheres are less spherical and have a rough surface. Both above-cited works confirm the results presented in this study. Different results were obtained by Irom et al. (17), who studied the morphology of chitosan microspheres with losartan potassium, which was obtained by emulsifying and solvent evaporation. They concluded that the increase of the polymer is adjusted to the improvement of particle morphology. It should be noted that they used a different methodology for obtaining the microspheres than in our study.

Analyzing the release profiles, we can see release of losartan potassium in acid medium despite the presence of Eudragit L30D55 (release at pH > 5.5 according to producer's information). This may be due to the interaction between losartan and methacrylic acid copolymer (Eudragit L30D55), since their negative charges result in mutual repulsion. It is also possible that during the spray-drying process, the glass transition temperature of the polymer is exceeded, which changes its properties. In a study conducted by Año (31) the release of *Vibrio cholerae* from microcapsules containing Eudragit L30D55 received by a spray-dried method was also found (about 3%). Fraiberg and Zhu (32) in their review point out that the release rate of the drug from the microspheres depends both on the type of polymer and the properties of the drug itself. The rate of release also depends on the weight of the polymer, drug distribution within the dosage form, encapsulating ability of polymer, crystallinity, and other factors.

The drug distribution in a polymer matrix also affects the release rate. Medicinal substance is rapidly released from a drug on the surface in the initial phase, and even distribution of the drug is provided by a controlled-release effect of the release profile, which is similar to the zero-order kinetics. Another parameter affecting the rate of release is the porosity of the matrix. More porous matrices release the drug faster than the less porous counterparts. First, the drug is eluted due to moistening the pores (rapid release phase), and then the extracellular matrix degrades slowly, and the active ingredient is slowly released (slower release phase). The release rate is also dependent on the size of the microspheres, the larger microspheres are, the slower drug release is. Preparations of modified, desirable release kinetics can be prepared by controlling the size of microspheres, which involves a suitable coating and linking different microspheres. In the literature, we can find reports stating that during spray drying unequal concentration distribution of dried ingredients can occur, which may contribute to a faster release of an active drug (33).

The studies on the release of losartan potassium from the microspheres to the artificial gastric and intestinal juices demonstrated that the active substance release from microspheres is dependent on the ratio of losartan potassium/ Eudragit L30D55, and the particle size. The obvious relation between the amount of polymer and the release rate of microspheres of losartan potassium could not be observed. Formulations 1 : 4 TEC and 1 : 5 TEC do not exhibit the effect of sustained release of the

active substance, but only slowing down the release in relation to the standard preparation. Formulation 1 : 1 TEC (R^2 = 0.994) has a dissolution profile similar to the ZOK formulas. It seems that the smaller and more spherical particles are, the more release profile resembles the zero-order kinetics. The amount of used Eudragit L30D55 has negligible effect on the release time of the drug from the microspheres and the percentage of its release, since the formulations 1 : 4 TEC and 1 : 5 TEC have the release time similar to the standard preparation without a sustained-release effect. Different results were obtained by Vidyadhara and colleagues (33) who released losartan potassium from the microspheres based on Eudragit S100 matrix, obtained by evaporation of the solvent in a ratio of 1 : 1–1 : 6. Analyzing the active substance release profile from the microspheres to a phosphate buffer at pH = 6.8, they stated that the increase of the amount of Eudragit S100 leads to extended time of the drug release. Kristmundsdottir et al. (34) have also observed that the amount of diltiazem released into the acceptor fluid decreases with an increased amount of the used polymer. Methods of obtaining microspheres in the cited studies, however, are significantly different from the methods of obtaining microspheres by a spray-drying process.

The addition of plasticizer to Eudragit L30D55 polymer matrix reduced the particle size of the microspheres, and extended release of the losartan potassium in a buffer of pH = 6.8. Between formulations with and without the plasticizer, we observed statistically significant differences. The addition of plasticizer slows down the release of losartan potassium of polymer matrices, and this modification of the release rate is preferred for long-term therapy.

Paluch and Tajber (35) have described a possibility of obtaining the amorphous systems during spray drying, which have a higher solubility and higher dissolution rate as compared to the crystalline form, which can lead to increased availability of the drug. Snajdrova and Dittrich (28) observed that the addition of plasticizer can lead to increased bioavailability of the drug. It is also possible that there is a change in the degree of the particles structure deformation, which may indicate an increased encapsulation efficiency.

Searching for possible causes of losartan potassium release in an artificial gastric juice, Raman spectra analysis was performed. Analysis of the Raman spectra showed that the peaks for losartan potassium in microsphere structures were shifted. The following values of Raman effect are changed from 1610 to 1613 cm^{-1}, from 1574 to 1578 cm^{-1}, from 1519 to 1527 cm^{-1} and from 1289 to 1295 cm^{-1}. In the case of losartan potassium electron binding forces substitutions may involve the aromatic ring or the imidazole ring, whereas in the case of Eudragit we can speak about the possibility of deformation of the C=O bond of carboxyl group (one free and one substituent esterified ether). The changes in the bonds in the losartan potassium and Eudragit L30D55 molecule can explain the change of Eudragit L30D55 properties, since release should occur at pH = 5.5 or in a more alkaline environment according to Evonik Industries product data. However, in our studies the release occurred at pH = 1.2.

The interaction between losartan potassium and Eudragit L30D55 seems probable because in their work Kubis and Musiał (36) indicated that there were interactions between the salts of active ingredients of alkaline nature, including acetates and sulpiride and doxepin hydrochloride salts of ephedrine and papaverine, and Eudragit L30D.

From the published data, it can be concluded that the release of an active substance from the microspheres occurs according to the first-order kinetics. Frequently, two phases can be observed – rapid and slow release phases (1, 37-40). Release kinetics of losartan potassium from examined microspheres demonstrated a profile of the 1- or 0-order kinetics. The release compatible with the 0-order kinetics appears to be advantageous because it gives the possibility to introduce the losartan potassium into the body at a constant speed.

The performed study confirmed that the use of a spray-drying technology to obtain microspheres meets the criteria of size and morphology of the microparticles.

CONCLUSIONS

The use of spray-drying techniques as well as the Eudragit L30D55 polymer matrix to obtain the microspheres containing losartan potassium makes it possible to obtain a product with the required particle morphology and particles size ensuring the release of the active substance up to 8 h.

The selection of appropriate parameters of spray drying allows to obtain microspheres with losartan potassium sizes of 2 – 12 μm, which enables the administration of losartan potassium in the form of microspheres not only orally but also parenterally (route of administration).

Authors declare no conflict of interest.

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ASSESSMENT OF GUAR AND XANTHAN GUM BASED FLOATING DRUG DELIVERY SYSTEM CONTAINING MEFENAMIC ACID

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Abstract: We aimed to assess guar and xanthan gum based floating drug delivery system containing mefenamic acid. Floating tablets of mefenamic acid were formulated with different concentrations of guar and xanthan gum *via* wet granulation method. The flow properties of granules that is: bulk density, tapped density, flow rate, Carr index, Hausner's ratio, compressibility index and angle of repose as well as physical parameters of the compressed tablets including: hardness, friability, thickness and swelling indices were determined and found to be good. Xanthan gum was superior to guar gum in maintaining drug release, but a combination of polymers was found to be the best for achieving sustained release up to 12 h due to the synergistic effect of both gums. Drug release mechanism was best explained by Korsmeyer-Peppas model. Fourier transform infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC) studies showed absence of any visible interaction. Stability studies at 40°C (75% RH) showed that the formulation was stable at elevated temperature. It can be concluded that floating tablets can be used as a sustained release matrix due to their superior characteristics.

Keywords: floating drug delivery system, xanthan gum, guar gum, kinetics, DSC, FTIR

Oral modified drug delivery system (DDS) has various limitations, that is, variable gastric residence time, non-uniform absorption and decreased gastric residence time of drugs that have an absorption window in the upper part of the gasterointestinal tract (1, 2). Gastro-retentive DDS increase the gastric residence time of drugs in stomach (3). Floating drug delivery system (FDDS) is a technique used to enhance gastric residence time, in which dosage form floats on the stomach contents for a prolong period of time (being less dense than gastric contents i.e., < 1.004 g/cm^3) and slowly release the drug without affecting the intrinsic emptying rate of stomach. FDDS are also called as hydro-dynamically balanced system (HBS) due to their ability to maintain low apparent density, whereas polymer hydrates and releases the drug slowly through gelled barrier (4, 5).

Mefenamic acid is a non-steroidal anti-inflammatory drug (NSAID), which is widely used as an analgesic and antipyretic specially used in the treatment of rheumatoid arthritis, dysmenorrhea, and osteoarthritis, mild to moderate pain, inflammation and fever (6). The daily dose is 1.5 g (7). It has a short half-life of 2 h so requires repeated administration (8). After oral administration of conventional dosage form, large amount of drug is absorbed and the excess metabolites bind covalently to renal macromolecules causing irreversible damage and necrosis at higher concentration. This effect is especially peculiar in patients with slow CYP 2CP metabolizer. This is a major drawback associated with a conventional dosage form of mefenamic acid (9). Thus, FDDS for mefenamic acid was needed to be developed so that only a controlled amount of mefenamic acid is released and the dosage form can

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be retained in stomach for a prolonged period of time.

Xanthan gum is a high molecular weight, biosynthetic, edible, anionic, hetero polysaccharide produced by Gram negative bacteria *Xanthomonas campestris* (10). Xanthan gum consists of glucose, mannose, and glucuronic acid (11, 12) and is used in different foods as thickener and stabilizer (13).

Guar gum is a natural, non-ionic, hetero polysaccharide obtained from endosperms of *Cyamopsis tetragonolobus* (family Leguminosae) (14). It is composed of sugars galactose and mannose. Its main backbone consists of linear chain of β-1,4-linked mannose units to which galactose units are attached at 1,6-linkage and form short side branches. Guar gum has high viscosity at low concentration, is seasily soluble in cold water. It is used as dietary fiber, thickening agent, emulsifying agent in food, cosmetics and pharmaceuticals (11).

The objective of this study was to formulate and characterize guar and xanthan gum based floating drug delivery system containing mefenamic acid.

MATERIALS

Mefenamic acid, lactose, magnesium stearate, talc, sodium bicarbonate, polyvinylpyrrolidone K30 and hydrochloric acid were gifted by Ideal Pharmaceuticals, Lahore- Pakistan. Guar gum, xanthan gum, citric acid, sodium chloride and potassium dihydrogen orthophosphate were purchased from Waheed Chemicals, Lahore, Pakistan.

METHODS

Formulation development

Wet granulation method was used to formulate the granules. All ingredients (Table 1) were passed separately through sieve 60# three times and mixed. A solution of PVP K30 (2%) in isopropyl alcohol was used as granulating/wetting agent. The granules were formed by passing through sieve #18 (15). These granules were dried at 50°C, lubricated with magnesium stearate and talc. A tablet punching machine (Rotary Tablet Press Machine Zp-17 series, Pakistan) was used to produce the tablets.

Characterization of granules

The pre-compression parameters were evaluated and were observed in the limit of good to passable flow ability for most of the formulations as per British Pharmacopoeia specifications.

Angle of repose

A sheet of paper was taken and its area was calculated by measuring length and width. Paper was weighed to calculate the grammage of paper by using the equation;

Grammage = wt. of paper/area of paper
(Unit of grammage is g/m^2) Eq. 1

Angle of repose was calculated by using the equation (16):

$$\tan \theta = \text{Height} / \text{Radius} \quad \text{Eq. 2}$$

$$\text{Therefore, } \theta = \tan^{-1} (\text{Height} / \text{Radius})$$

Flow rate

The flow rate was measured by pouring a fixed amount of granular mass into a funnel and measuring the time (by stop watch) required to flow through the funnel orifice (16). It was calculated by the following equation:

Flow rate = weight of granules / time in seconds Eq. 3

Bulk density

A specific amount (50 g) of pre-sieved sample was introduced into a 100 mL graduated cylinder

Table 1. Different relative amount of each ingredient in mg unit (w/w).

Ingredients	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Mefenamic acid	250	250	250	250	250	250	250	250	250	250
Lactose	179.2	121.2	63.2	179.2	121.2	63.2	63.2	121.2	179.2	306.8
$NaHCO_3$	58	58	58	58	58	58	58	58	58	-
Citric acid	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	-
Guar gum	58	116	174	-	-	-	87	58	29	-
Xanthan gum	-	-	-	58	116	174	87	58	29	-
PVP K30	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6	11.6
Mg stearate	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Talc	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Total weight (mg)	580	580	580	580	580	580	580	580	580	580

and volume of powder was noted as it is. Bulk density was calculated by the equation:

Bulk density = weight of powder/ bulk volume Eq. 4

Its unit is g/cm^3 (17).

Tapped density

A fixed amount (50 g) of pre-sieved sample was introduced into 100 mL graduated cylinder and was allowed to fall from a height of 2.5 cm and for many times until there was no change in volume. Tapped volume was measured by following the equation.

Tapped density = weight of granules/ tapped volume Eq. 5

Its unit is g/cm^3 (17).

Granules/Powder flow properties

Compressibility index

It's an indirect measure of bulk properties. Compressibility index was determined by the Carr's compressibility index (18). It was expressed in percentage and given by the following equation:

Compressibility index = (tapped density - bulk density/tapped density) × 100 Eq. 6

Hausner's ratio

It was given in percentage and calculated by the following equation (18):

Hausner's ratio = tapped density/bulk density Eq. 7

Characterization of prepared tablets

Weight variation test

Twenty tablets were randomly selected and weighed accurately. The weight of all tablets was checked against the lower and upper limit. The batches passed the test if not more than 2 tablets were outside the allowed limit and none deviated twice the limit (19).

Tablets thickness and diameter

Ten tablets were randomly selected. A Vernier caliper was used to determine thickness and diameter of each tablet. So lower and upper limit was calculated and thickness of all tablets was compared with the mean value (19).

Drug content uniformity

Twenty tablets were taken randomly from each batch, weighed and powdered. Powder that contained 250 mg of mefenamic acid was taken and dissolved in 100 mL of absolute ethanol. It was titrated with 0.1 sodium hydroxide using phenol red as an indicator. Each mL of 0.1 M sodium hydroxide is equivalent to 0.2413 g.

% of mefenamic acid = volume of NaOH used × factor × 100/weight of sample Eq. 8

Assay limit is 95-105% (British Pharmacopoeia 2009).

Tablet friability

Twenty tablets were randomly selected, weighed (W1) and placed in tablet friability test apparatus. The drum was adjusted to rotate at a speed of 25 rpm dropping the tablets from a height of 6 inches with every revolution. It was allowed to rotate for 4 min. After this time tablets were removed, dusted and weighed again (W2) (19). The percentage weight loss was calculated by the equation:

% Friability = (initial weight - final weight/ initial weight) × 100 Eq. 9

Tablet swelling ability

A tablet from each batch was weighed (W1) and placed in a glass beaker containing 200 mL of 0.1 M hydrochloric acid. After regular time interval, the tablet was removed and excess surface liquid was dried by filter paper and weighed (W2) (20). The swelling index was calculated through an equation:

S.I. = W2 -W1/W1 (W2 = weight after swelling, W1= weight before swelling) Eq. 10

Tablet floating behavior

Tablets were placed in a beaker containing 200 mL of 0.1 M hydrochloric acid. The time duration taken by tablet to come to the surface was determined (the floating lag time). The total time during which the tablet remained buoyant (called as total floating time - TFT) was recorded (21).

***In vitro* dissolution**

USP dissolution apparatus type-II (paddle method) was used to perform drug release studies of prepared floating tablets at 37 ± 0.5^{o}C and 100 rpm speed. Tablets were placed into 900 mL of hydrochloric acid solution (pH 1.2) for 2 h. A sample of 5 mL was withdrawn from the dissolution apparatus at regular suitable time intervals and filtered. Samples were analyzed by a spectrophotometer at 285 nm wavelength. After removal of each sample of 5 mL from dissolution apparatus, fresh medium was added to replace the fluid loss. The same procedure was done in phosphate buffer solution (pH 6.8) for 10 h (5, 28).

Application of kinetic models on drug release profile

The dissolution profile of all formulations was fitted to zero order, first order, Higuchi and

Korsmeyer-Peppas models. A software called DDSolver was used to estimate the kinetic models. The model that exhibited the highest correlation coefficient value was considered to be the best model describing the release kinetics (24).

Compatibility studies

Fourier transform infrared spectroscopy (FTIR)

The compatibility of mefenamic acid and polymers was studied through FTIR analysis (22). The FTIR study was performed using Thermo Nicolet FTIR 6700 apparatus and potassium bromide and data were analyzed by using software. For analysis, very small quantity of sample was placed on the lens of equipment directly and pressure was applied through screw up to 12 marks. The spectrum was recorded between 4000-400 cm^{-1}. FTIR spectra of pure mefenamic acid, pure xanthan gum, pure guar gum and mefenamic acid with all other excipients were determined.

Differential scanning calorimetry (DSC)

Thermal analysis was performed to find the compatibility of active ingredient and polymers (21). It was performed with differential scanning calorimeter AT Q2000 (New Castle, Delaware, USA) and data were analyzed by TA (Thermal Analysis) Universal Analysis software. The weighed amount (10-12 mg) of sample, i.e., mefenamic acid was sealed in aluminium pans. The analysis was performed at a nitrogen flow of 10 mL/min and scanning was done at 10°C per min from 50 to 250°C. A blank aluminium pan was placed as reference.

Physical stability studies

Physical stability studies were conducted according to International Conference on Harmonization Guidelines (ICH). Tablets from best suitable formulation were closed in polyethylene bottles and placed in a desiccator containing saturated solution of sodium chloride (75% RH). The desiccator was placed in an oven at 40°C for 3 months. After suitable specified time intervals, the tablets were examined for any statistical difference in their hardness, matrix integrity (physical appearance), and floating characteristics.

RESULTS AND DISCUSSION

Characterization of granules for each batch was done separately (Table 2). Angle of repose was found to be in the range of 26.55 ± 1.34 to 34.32 ± 2.56° that indicates excellent to good flow properties of granules (B.P 2009). Bulk density was found to be in the range of 0.672 ± 0.009 to 0.697 ± 0.0005 g/cm^3. The tapped density range was found to be 0.779 ± 0.008 to 0.808 ± 0.008 g/cm^3. F1 and F4 had the lowest tapped density, whereas F6 had the highest value of tapped density. Carr's index value range was found to be from 11.98 ± 1.65 to 14.92 ± 1.75 that indicated good flow properties. Similarly, Hausner's ratio also confirmed the good flow of granules with value ranging from 1.2 ± 0.01 to 1.17 ± 0.02. Flow rate range was 8-9 g/s. (B.P. flow rate limit 7-10 g/s). The flow properties of all batches of granules indicated that granules don't need any agitation support to enhance their flow rate.

Table 2. Pre-compression parameters of granules.

Formulation	Parameters				
	Angle of repose (θ)	Bulk density (g/cm^3)	Tapped density (g/cm^3)	Carr's index	Hausner's ratio
F1	26.55 ± 1.34	0.690 ± 0.006	0.779 ± 0.008	12.31 ± 0.94	1.12 ± 0.01
F2	31.03 ± 1.56	0.693 ± 0.010	0.786 ± 0.011	13.49 ± 2.07	1.13 ± 0.02
F3	28.76 ± 2.1	0.697 ± 0.0005	0.788 ± 0.008	12.62 ± 0.88	1.13 ± 0.011
F4	32.56 ± 0.56	0.695 ± 0.01	0.779 ± 0.008	12.72 ± 1.75	1.12 ± 0.015
F5	34.32 ± 2.56	0.684 ± 0.009	0.786 ± 0.011	11.98 ± 1.65	1.14 ± 0.001
F6	28.91 ± 1.81	0.692 ± 0.004	0.808 ± 0.008	12.31 ± 0.94	1.16 ± 0.02
F7	27.32 ± 3.1	0.682 ± 0.004	0.800 ± 0.002	14.89 ± 2.07	1.17 ± 0.02
F8	28.43 ± 2.49	0.672 ± 0.009	0.7926 ± 0.01	13.72 ± 0.88	1.17 ± 0.011
F9	32.03 ± 1.52	0.683 ± 0.005	0.791 ± 0.011	13.52 ± 1.75	1.15 ± 0.02
F10	27.34 ± 2.84	0.676 ± 0.120	0.793 ± 0.01	14.92 ± 1.75	1.17 ± 0.02

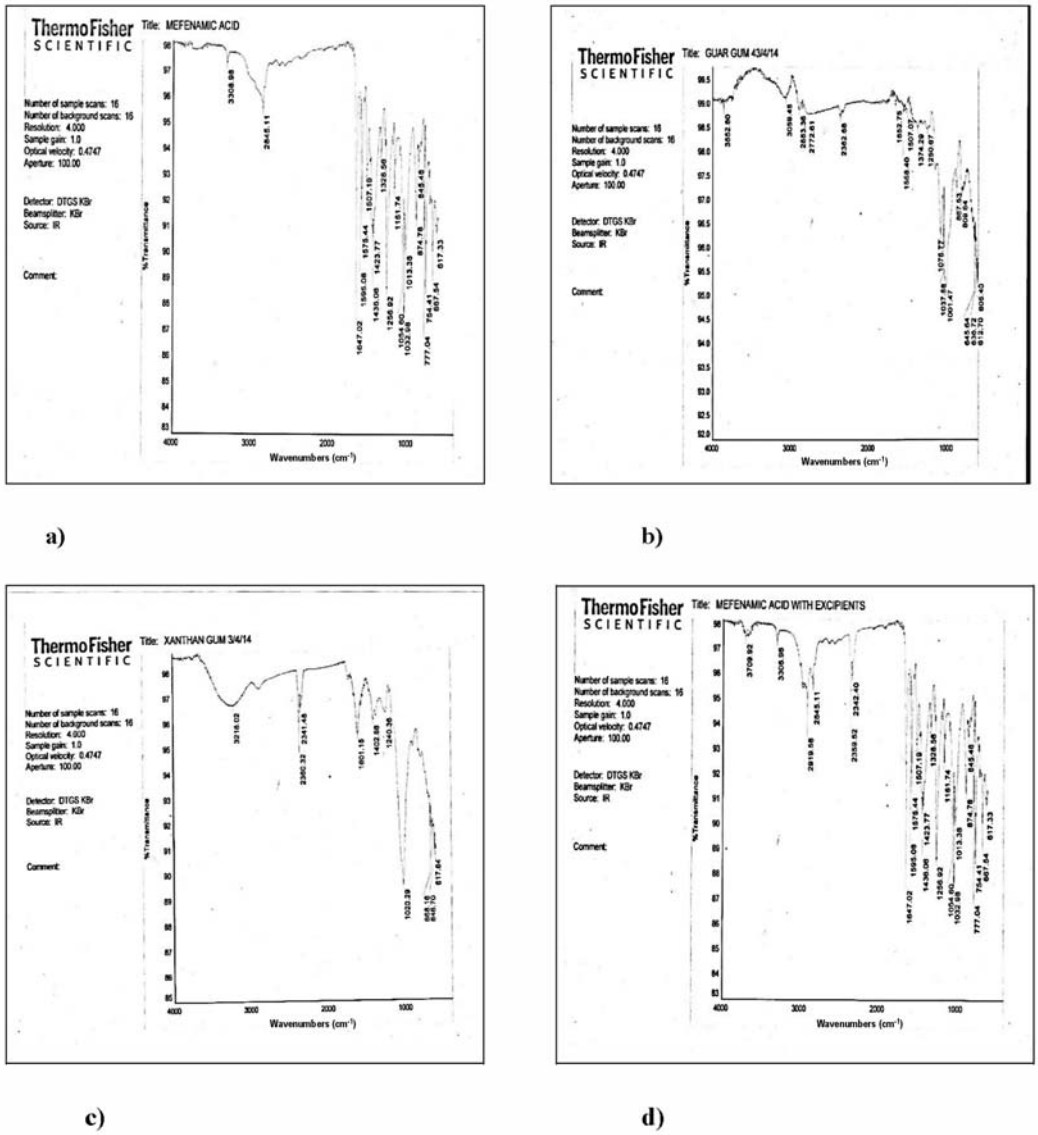


Figure 1. FTIR spectra (4000-400 cm^{-1}) of **a)** mefenamic acid, **b**) guar gum, **c)** xanthan gum and **d)** mefenamic acid with xanthan gum, guar gum and other excipients

After compression, flat and off white colored tablets were resulted (Table 3). The weight variation range was 578 ± 1.45 (F8) to 583 ± 2.43 mg (F10). All batches were within the permitted range of weight variation ±5% of official criteria (B.P. 2009). Percentage drug content was in range from 98.02 ± 3.79 (F4) to 4.45 ± 0.072 (F9). All tablets showed more than 98% active drug content that confirmed uniformity of drug contents. This uniformity is due to uniform mixing of active ingredient with excipient during granulation. Thickness and diameter showed a uniform trend. Thickness range was found to be 4.29 ± 0.05 (F7) to 4.45 ± 0.072 mm (F9). Diameter range was found to be 10.13 ± 0.05 (F6) to 10.31 ± 0.10 mm (F2). Friability results showed that the tablets had good hardness and mechanical strength. F6 had the highest percentage friability of 0.54 ± 0.04%.

The swelling index is an indicative of the dissolution rate of tablet and determines the mechanism of drug release (20). Swelling index (Table 4, Fig. 3) showed that tablets with xanthan gum had generally

higher swelling ability and swelling index than tablets that contained guar gum. This effect may be attributed to the inherent ability of xanthan gum to produce a more viscous solution. The reason behind this phenomena observed was that xanthan gum contained anionic side chain (due to presence of the carboxylate on glucoronic acid side chain residue) that is responsible for greater hydration and more swelling ability than guar gum (11). F1, F2 and F3 showed S.I. of 147, 151 and 181%, respectively, whereas F4, F5 and F6 showed S.I. of 203, 226 and 239%, respectively. A direct relationship was observed between swelling index and quantity of polymer gum. Moreover, xanthan gum showed synergism in the presence of galactomannan gums (guar gum) that's why tablets containing a mixture of xanthan and guar gum showed the highest swelling index (11, 19). F7, F8 and F9 showed S.I. of 308, 254 and 241% that contained 30% of the mixed gum polymer. F10 (blank formulation) did not show any swelling sign because absence of polymer. The swelling trend could be summarized as rank order of mixed polymer > xanthan gum > guar

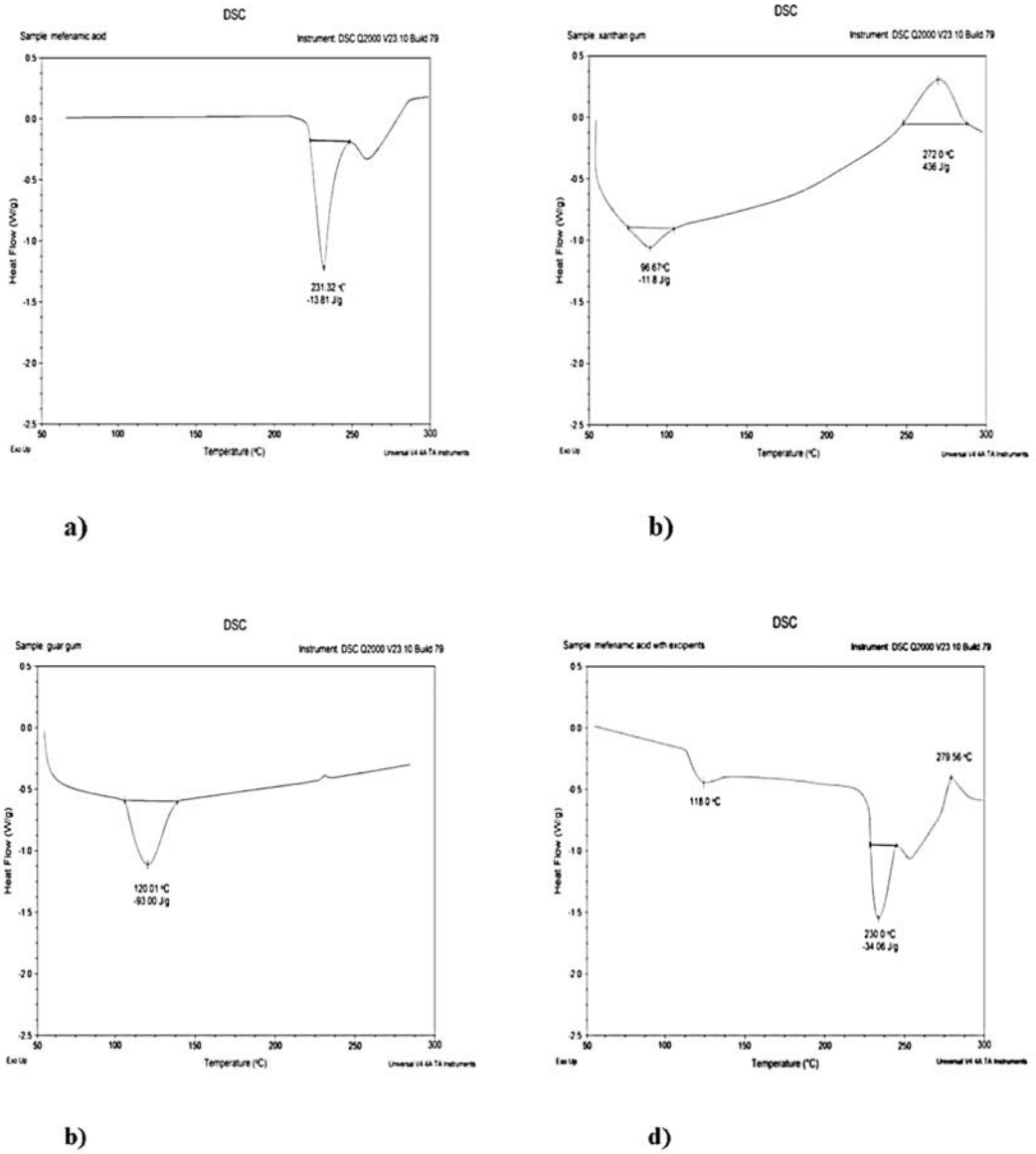


Figure 2. DSC thermograms of **a)** mefenamic acid, **b)** xanthan gum, **c)** guar gum and **d)** mefenamic acid with excipients

Table 3. Different parameters of tablets.

Formulation	Weight variation (mg)	Friability (%)	Hardness (kg/cm^2)	Drug content (%)
F1	580 ± 1.19	0.42 ± 0.03	5.6 ± 0.47	98.89 ± 2.65
F2	579 ± 2.89	0.48 ± 0.08	6.4 ± 0.23	100.59 ± 2.09
F3	583 ± 1.89	0.49 ± 0.08	6.3 ± 0.25	98.32 ± 2.92
F4	581 ± 2.1	0.45 ± 0.02	5.6 ± 0.34	98.02 ± 3.79
F5	581 ± 2.00	0.47 ± 0.05	7.0 ± 1.01	98.99 ± 3.18
F6	578 ± 1.67	0.54 ± 0.04	5.7 ± 1.00	99.15 ± 4.680
F7	582 ± 1.76	0.53 ± 0.09	6.01 ± 0.77	100.11 ± 2.38
F8	578 ± 1.45	0.49 ± 0.04	6.60 ± 0.45	99.14 ± 2.37
F9	580 ± 1.12	0.46 ± 0.05	6.24 ± 0.67	99.15 ± 3.680
F10	583 ± 2.43	0.42 ± 0.03	5.6 ± 0.78	100.11 ± 2.65

Table 4. Swelling behavior of tablets F1-F9.

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	17	18	20	24	31	35	34	27	22	0
2	51	71	75	85	90	145	167	116	125	0
3	105	99	118	169	185	210	221	192	165	0
4	110	101	136	175	198	212	261	221	176	0
5	114	110	152	179	206	214	271	236	198	0
6	118	134	169	182	207	218	287	239	201	0
7	121	138	171	185	209	221	291	242	204	0
8	126	140	171	186	211	226	293	246	208	0
9	131	139	174	192	211	229	294	248	212	0
10	136	141	175	195	218	215	301	251	218	0
11	142	146	178	198	221	226	303	252	223	0
12	147	151	181	203	226	239	308	254	241	0

gum. The initial rate of swelling is rapid and then there is a slow increase in swelling because initially hydration rate is rapid, later on hydration rate decreases and there is dissolution of outer most gelled layer of hydrophilic polymer (11).

Tablets containing xanthan gum generally showed longer total floating time. It ranged from 12 to 24 h. F7 had TFT 24 h due to the synergistic effect of xanthan and guar gum, higher amount of polymer that made dosage form excellently buoyant due to maximum swelling (Table 5, Figure 4). F10 (blank formulation) did not float because of lack of any polymer.

For dissolution: 900 mL dissolution medium (disintegration test medium No. 1 (pH 1.2) and No. 2 (pH 6.8) as specified in Japanese Pharmacopoeia XI and as corresponding to USP XXI, paddle method at 37°C at 100 rpm (5, 28). Calibration curve was developed in phosphate buffer 6.8 to determine the concentration of mefenamic acid in the unknown sample. A linear relationship was observed between the concentration of mefenamic acid and absorbance.

At the end of 12 h, F1 showed the highest drug release - 97.92%. F2, F3, F4, F5, F6, F7, F8 and F9 showed 93.1, 90.1, 91.67, 89.27, 85.45, 73.8, 88.1 and 91.5%, respectively, shown in Table 6. F7 showed the best sustained release behavior (73.18%). There was an inverse relationship between the amount of polymer added and drug

release. This phenomenon is due to the fact that higher concentration of swellable polymer produced thick outer gelatinous layer that increases the diffusion pathway for both the drug and penetrant. Due to this increase in diffusion pathway length, penetration of dissolution media into the tablet interior region is prohibited that results in more sustained release. Moreover, there is the slow erosion of outer gelled structure due to the high amount of polymer (19). Xanthan gum containing tablets showed better sustained release profile than guar gum. F3, F4 and F5 showed 91.67, 89.27 and 85.45% release after 12 h. The reason behind this observed phenomenon was that xanthan gum particles were hydrated rapidly at the start due to the presence of anionic side

Table 5. Floating behavior of tablets F1-F9.

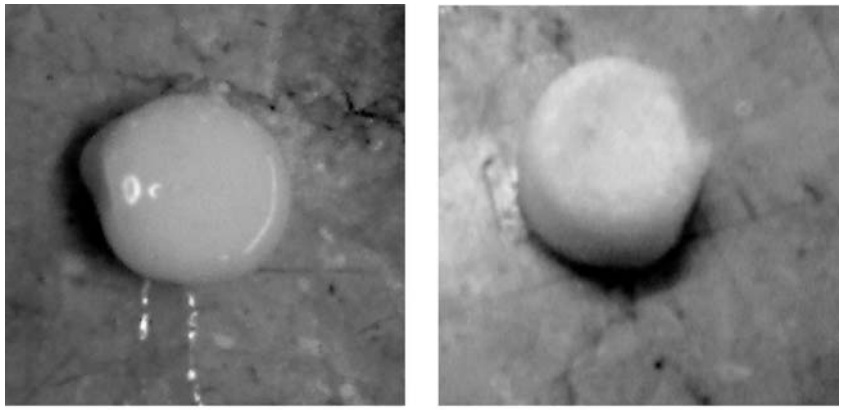
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Lag time	4 min 10 s	4 min 30 s	4 min 40 s	4 min 30 s	3 min 5 s	5 min	3 min 30 s	4 min	5 min	Do not float
TFT (h)	12	14	18	12	16	20	24	20	14	Do not float

Table 6. % Cumulative drug release profile of F1-F10.

Time (h)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
1	5.73	6.25	5.42	7.63	7.34	6.54	6.15	7.54	8.85	37
2	9.76	14.4	16.5	10.4	9.61	10.11	13.99	11	11.5	74
3	29.53	26.6	24.7	14	12.41	27.34	19.67	18.5	27.3	97
4	40.45	35.6	30.6	29.04	25.34	38.43	24.53	24.1	39.6	97
5	47.06	46	51	36.02	35.24	40.13	34.93	35.11	57.01	97
6	55.98	57.5	60.1	45.76	50.12	50.43	41.94	49.4	60.54	97
7	71.76	70.8	70.5	58.16	57.51	63.35	46.26	55.5	71.8	97
8	74.59	77.9	73	66.57	63.25	72.35	50.52	72.7	80.9	97
9	80.11	83.5	79.1	74.54	73.13	75.56	52.73	74.5	83.5	97
10	85.11	88.8	85	81.74	80.23	80.56	57.16	80.5	85.5	97
11	91.69	92.8	88.3	87.56	86.26	83.14	67.40	85.8	90.00	97
12	97.92	93.1	90.1	91.67	89.27	85.45	73.18	88.10	91.5	97

Table 7. Pharmacokinetic modelling (F1-F9).

Model	Zero order		First order		Higuchi		Hixson-Crowell		Korsmeyer-Peppas	
Formulation	R^2	K_0	R^2	K_1	R^2	K_H	R^2	K_{HC}	R^2	n
F1	0.9681	8.772	0.9155	0.156	0.8388	24.968	0.9552	0.044	0.9749	0.894
F2	0.9653	8.813	0.9128	0.156	0.8364	25.088	0.9548	0.044	0.9723	0.894
F3	0.9495	8.527	0.9262	0.150	0.8409	24.334	0.9603	0.042	0.9624	0.860
F4	0.9814	7.896	0.8803	0.125	0.7724	22.173	0.9231	0.036	0.9854	1.096
F5	0.9750	7.740	0.8798	0.121	0.7650	21.720	0.9207	0.035	0.9794	1.102
F6	0.9581	8.021	0.9374	0.135	0.8409	22.868	0.9660	0.038	0.9683	0.874
F7	0.9828	6.171	0.9704	0.089	0.8517	17.579	0.9839	0.026	0.9890	0.899
F8	0.9722	7.846	0.8863	0.125	0.7777	22.091	0.9264	0.036	0.9735	1.052
F9	0.9243	8.868	0.9277	0.164	0.8512	25.415	0.9614	0.046	0.9510	0.809



a) b)

Figure 3. Floating drug delivery system after **a)** 6 h swelling and **b)** 10 h swelling

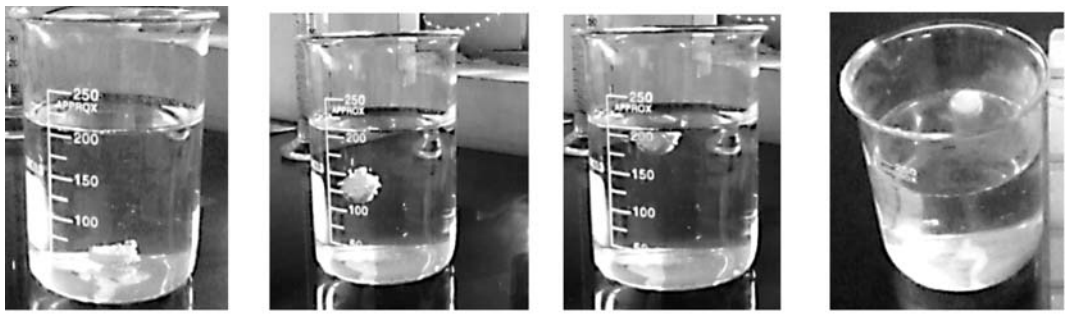


Figure 4. Floating tablets at given time intervals

chain. The hydrated particles coalesce and form continuous viscoelastic matrix that fills the interstices maintaining the integrity of the tablets and retarding further penetration of medium (23). F7, F8 and F9 that contained mixed polymer showed much better sustained release profile. F7 showed maximum controlled release of 73.18% after 12 h. This showed that tablets containing matrix composed of mixed polymer gave better sustained release of drug release due to their synergistic effect (11).

Kinetic modelling was applied to dissolution data obtained for F1-F9 (Table 7). F1, F2, F3, F4, F5, F6, F7, F8 and F9 showed R^2 value of 0.9749, 0.9723, 0.9624, 0.9854, 0.9794, 0.9683, 0.9890, 0.9735 and 0.9510, respectively, for the Korsemeyer-Peppas model. All formulations showed the highest R^2 value in the Korsemeyer-Peppas model. F1 and F2 had n value of 0.894 and 0.894, so mechanism of drug release from these formulations was case II transport i.e., close to zero order mechanism. This phenomenon could be attributed to structural changes (relaxation of polymer) induced in the polymer by penetrant (10). F7 also showed case II transport with R^2 value of 0.899. F4, F5, F8 had n value of 1.09, 1.102 and 1.052 that showed that the drug release from these formulations followed super case II transport mechanism (24). The R^2 values of all formulations are given in Table 7. F3, F6 and F9 had n value of 0.860, 0.874 and 0.809, respectively, that showed that drug release from these formulations were followed through anomalous diffusion. The drug release mechanism was found to be mixed type. Both diffusion and erosion of polymer were involved. Thua, the drug release mechanism from all formulations could be best explained through Korsemeyer-Peppas model.

To check physicochemical interactions between the drug and its excipients FTIR spectra

were determined (25) on pure samples of mefenamic acid, guar gum, xanthan gum and on a mixture of mefenamic acid with excipients (Fig. 1). Main peaks were obtained at 1647, 1574, 1510, 1256, 1161 and 754.13 cm^{-1}. Similar peaks were reported in B.P. 2009 for pure mefenamic acid. The peak at 1647.02 cm^{-1} represented the stretching of (C=O) carbonyl group, peak at 3308.98 cm^{-1} showed N-H stretching. Intense band at 1575.44 cm^{-1} corresponded to N-H bending and at 1510 cm^{-1} the characteristic peak represented C = C stretching. At 1038 cm^{-1} the peak represented the presence of phenyl group. The peak at wave number 754 cm^{-1} corresponded to aromatic stretching.

Guar gum gave peak at 3059 cm^{-1} (due to OH stretching) and 2883 cm^{-1} (due to aliphatic C-H stretching). Xanthan gum gave peak at 3218 cm^{-1} (due to OH stretching) and at 2360 cm^{-1} (due to C-H stretching). There was no major shift in peaks in the FTIR of mefenamic acid with its excipients. All characteristic peaks of mefenamic acid were preserved and present. So it was concluded that there was no interaction between mefenamic acid and the excipients.

Differential scanning calorimetry (DSC) is a powerful method for measuring directly the thermodynamic parameters and much used for the study of the stability in the process of product development (26). DSC studies were done to check physicochemical interactions between the drug and excipients at 50-300^{o}C using DSC Q2000 (Fig. 2). An endothermic peak was observed at 231.32^{o}C in the DSC graph of mefenamic acid due to melting of mefenamic acid. DSC graph of xanthan gum showed an endothermic peak at 96.67^{o}C and exothermic peak at 270^{o}C. DSC of guar gum showed an endothermic peak at 120^{o}C.There was no major shift in the peaks of pure drug. The DSC of mefenamic acid with its all ingredients showed endothermic peak at 230^{o}C. The reported melting point range of mefenamic acid in official books was 230-231^{o}C (27). Thus, no interaction was found between mefenamic acid and excipients.

The results of stability studies, indicated that the tablets did not show any physical changes (color change, friability and hardness), lag time and TFT during the study period kept at 25^{o}C ± 75% RH and the drug content (n = 3; mean ± SD) was found above 98% at the end of 90 days (d) (0 d: 100.11%; 30 d: 98.78%; 60 d: 98.45%; 90 d: 98.11%). Similar results were found at accelerated condition (40^{o}C ± 75% RH) and the drug content (n = 3; mean ± SD) was found above 98% at the end of 90 d (0 d: 100.11%; 30 d: 98.34%; 60 d: 98.20% 90 d: 98.01%). However, real time stability studies for a period of 2 years are required to establish the stability of the developed product.

CONCLUSION

The combination of xanthan and guar gum gave better sustained release as compared to either of gum used alone. Mixed polymer (30% - 15% xanthan +15% guar) showed the best sustained release up to 12 h and could be investigated in further *in vivo* studies.

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STUDIES OF THE CRYSTALLINE FORM OF CEFUROXIME AXETIL: IMPLICATIONS FOR ITS COMPATIBILITY WITH EXCIPIENTS

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Abstract: Amorphous and crystalline forms of cefuroxime axetil were identified and characterized using DSC, XRPD, SEM, FT-IR and Raman spectroscopy. Based on the results of chromatographic studies, changes in the kinetic mechanism and rate of degradation of the crystalline form of cefuroxime axetil in binary systems with excipients were also evaluated. The findings suggest that the mechanism of degradation of cefuroxime axetil in such systems depends on two factors: the applied excipient and storage conditions. Cefuroxime axetil in combination with magnesium stearate, croscarmellose sodium and crospovidone, microcrystalline cellulose, aerosil is decomposed according to the first-order reaction model in dry air as well as at an increased relative air humidity, which may be associated with non-catalytic interactions between the active pharmaceutical ingredient and the excipients. However, in the presence of mannitol, under elevated humidity conditions (RH ~ 76%), the degradation of cefuroxime axetil follows the autocatalytic model. According to ESP maps, computed binding energies and HOMO – LUMO gaps, differences of degradation curves between cefuroxime axetil – mannitol and other investigated systems were explained. This study of the polymorphic transformation of the crystalline form of cefuroxime axetil and its binary systems with excipients after exposure to increased temperature and humidity indicated a conversion towards the amorphous form or the coexistence of both forms.

Keywords: cefuroxime axetil, polymorphism, compatibility, stability, polymorphic transformation

Cefuroxime axetil (CA) (Fig. 1) belongs to the second generation of cephalosporins characterized by a broad spectrum of antibacterial activity and is used as an oral pro-drug (1). CA contains various isomeric centers and occurs in crystalline and amorphous forms. The stereochemical properties of CA are essential for its antibacterial activity and affinity to biological membranes. A syn-geometric isomer demonstrates considerable resistance to β-lactamases and is easily transported while an anti-isomer of CA is deactivated by cephalosporinases (2, 3). The presence of polymorphic forms can influence the solubility and bioavailability of CA (4). The Abbreviated New Drug Application (ANDA) has approved CA as an amorphous dispersion or an amorphous/crystalline mixture following the expiry of patent protection and the appearance of generic products (5). It is well known that solid-state polymorphic transformation of an API (active pharmaceutical ingredient) can occur during preformulation studies and polymorphism of pharmaceutical substances can be connected with their different physical, chemical and mechanical properties as well as with differences in their bioavailability (6-9). The reasons of changes of bioavailability of amorphous form of drug in comparison to crystalline one are different apparent aqueous solubility and dissolution rate. In the result, polymorphs of the same drug can have different processability and stability which can lead to differences in their bioavailability. The most frequent and important factors which can induce polymorphic conversion are humidity and temperature (10). In the case of CA, it was suggested that the crystalline content in the mixture of CA may "seed"

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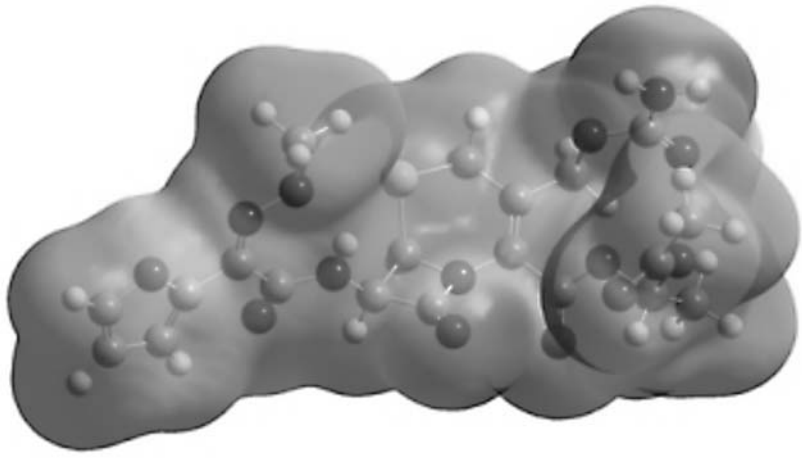

Figure 1. Electrostatic potential map of cefuroxime axetil

crystallization of the amorphous form and so increase the content of the crystalline form that is known to be less soluble and bioavailable (11). The literature does not provide reports allowing evaluation of behavior of crystalline form of CA in the presence of excipients commonly applied for preformulation studies. Only polarizing microscopy has been proposed for analysis of polymorphic forms of cefuroxime axetil (12). Researchers however did not supply data necessary to study the polymorphism of CA during the manufacture of batch-to-batch products as well as for quality control of drug throughout its shelf-life.

The previous stability studies of CA focused only on analysis of its amorphous form, including evaluation of its kinetic degradation, adsorption and photoisomerization of diastereoisomers (13-16). The stability of crystalline form in regards to the content of diastereoisomers and evaluation of its kinetic degradation in the presence of excipients have not been reported yet.

The work aimed to examine the degradation of the crystalline form of cefuroxime axetil in the presence of selected excipients and to analyze its polymorphic transformation *via* the stability study.

EXPERIMENTAL

Materials and reagents

The CA polymorphs were provided by the Institute of Biotechnology and Antibiotics in Warsaw, Poland. The excipients were: mannitol, microcrystalline cellulose, aerosil, magnesium stearate, croscarmellose sodium, crospovidone and they were supplied by Comet Innisfil, Canada. Other chemicals and reagents were obtained from Merck KGaA (Darmstadt, Germany) and were of chromatographic grade. High quality pure water was prepared using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120). Physical binary mixtures of CA : each excipient alone (1 : 1 mass/mass ratio) obtained by gridding in the agate mortar were also studied.

Scanning electron microscopy (SEM)

Prior to the study all samples were coated with mixture of gold and palladium in Polaron Range SC7620 Sputter Coater. Time of coating was set to 135 s. The surfaces of crystalline and amorphous forms of CA were observed with the use of Hitachi S-3000N Scanning Electron Microscope.

Raman spectroscopy (RS)

The Raman scattering spectra of crystalline and amorphous forms of CA were investigated in the spectral range of 100–3200 cm^{-1}. The nonpolarized Raman spectra were recorded in the back-scattering geometry using inVia Renishaw micro-Raman system. As an excitation light we used the near infrared laser operating at 785 nm. The laser beam was tightly focused on the sample surface through a Leica 50x LWD microscope objective (LWD - long working distance) with numerical aperture (NA) equals to 0.5 leading to a laser beam diameter about 2.0 μm. To prevent any damages of the sample an excitation power was fixed at 1.0 mW.

The position of the microscope objective with respect to the thin layer was piezoelectrically controlled. The reference position (level 0) was

assumed for the laser spot focused on the surface of sample. The inVia Raman spectrometer allowed for recording the Raman spectra with the spatial resolution of about 1.0 μm.

Differential scanning calorimetry (DSC)

DSC analysis of crystalline and amorphous forms of CA were performed by using DSC 1 Star System (Mettler Toledo, Zurich, Switzerland). The samples were sealed in aluminum cells with pierced lids. The measurements were performed in nitrogen atmosphere within the temperature range from 313 K to 613 K, with a heating step 283 K/min. The cell constant calibration method was applied to analysis of the DSC patterns.

X-ray powder diffraction (XRPD)

X-ray powder diffraction studies of crystalline and amorphous forms of CA were carried out by the use of modified HZG-4 powder diffractometer using the CuKa radiation. The powdered samples subjected for X-ray diffraction analysis were placed on vertical polycarbonate holders. X-ray diffraction data were collected in the $2\theta = 4\text{-}60^{o}$ range at the scanning rate 0.02 s^{-1} in room temperature. The diffraction data were processed and analyzed using Origin software.

Ultra-performance liquid chromatography (UHPLC)

Chromatographic studies were performed on a Thermo Scientific UHPLC - UltiMate 3000 system. Diastereoisomers of CA were separated with a Kinetex C-18 (100 mm × 2.1 mm, 1.7 μm) column as a stationary phase and a mixture of 0.1% formic acid : methanol (88 : 12, v/v) at the flow rate of 0.7 mL/min as the mobile phase. The wavelength of the DAD detector was set at 278 nm. Separation was carried out at 313 K. The injected volume was 5.0 μL. The components of mobile phase and samples solutions were filtered through 0.2 μm nylon membranes.

Theoretical studies

For visualization purpose, ChemBio3D and GaussView applications were used. Geometry optimizations were carried out based on density functional theory (DFT) using hybrid functional Becke, 3-parameter, Lyn-Yan-Parr (B3LYP) with 6-31G(d,p) standard basis set. Computational studies were conducted in order to support experimental results of investigation of degradation of cefuroxime axetil – excipient systems. The changes observed on degradation curves were explained with utilization of molecular modelling. Quantum mechanical calculations were carried out with Gaussian 09 package. The separated molecules of cefuroxime axetil and excipients were optimized with DFT before docking with AutoDock Vina 1.1.2. Because of significant size of basis set of API – excipient complexes, semiempirical PM6 method was used to provide reasonably accurate geometry optimization and bonding energies calculation. Systems which involved polymers were modelled as monomer – API system for sake of computational feasibility. Electrostatic potential maps were computed basing on single point energy calculation with DFT B3LYP method with 6-31+G(d,p) basis set. Diffuse basis functions were added in order to accurately calculate intermolecular bonding energy (17).

Kinetic and compatibility studies

For the forced aging test, 5.0 mg samples of CA were weighed into 5.0 mL vials. To evaluate their stability in an increased air humidity, they were placed in heat chambers at 353 K in desiccator containing saturated solution of inorganic salt – sodium chloride (~76 % RH). To evaluate the stability of CA in dry air, the vials were immersed in a sand bath placed in heat chambers at 383 K. At specified time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and their contents were dissolved in the mixture of acetonitrile : water (50 : 50, v/v) and injected into the chromatographic column at a concentration 0.2 mg/mL. The compatibility and stability of binary mixtures of CA with commonly used excipients (mannitol, microcrystalline cellulose, magnesium stearate, croscarmellose sodium, aerosil, crospovidone) were investigated in the same conditions as bulk substance (~76 % RH at 353 and 0% RH at 383 K). The same solvent was applied and final concentration 0.2 mg/mL was also achieved.

RESULTS AND DISCUSSION

Solid-state identification and characterization of crystalline form of CA

At the first stage of our studies, the confirmation of crystalline form of CA was conducted. DSC, SEM, XRD, FT-IR and RS were proposed for identification of crystalline and amorphous forms of CA. In our previous paper we confirmed possibility of application of vibrational spectra (FT-IR and Raman spectroscopy) for differentiation analysis of amorphous and crystalline forms of CA (18). In DSC analysis, the crystalline form of CA exhibited single peak at 400.18 K, corresponding to its melting point. On the

other hand, the amorphous form exhibited an endothermic transition with peak at 359.98 K (Fig. 2).

As shown in Figure 3, the characteristic hexagonal-type crystals were observed in samples of crystalline form of CA. On the contrary, morphologic analysis of amorphous form of CA indicated the presence of uniformly distributed spherical shaped aggregates.

The X-ray diffraction data collected for crystalline and amorphous forms of CA are presented in

Table 1. Observed rate constants of the degradation of crystalline and amorphous forms of cefuroxime axetil.

Polymorphic form	Condition of degradation	
	RH = 0%, T = 383 K $(k \pm \Delta k)$ [s^{-1}]	RH ~ 76%, T = 353 K $(k \pm \Delta k)$ [s^{-1}]
Crystalline form	$(5.24 \pm 2.81) \times 10^{-6}$	$(8.70 \pm 0.13) \times 10^{-6}$
Amorphous form	$(2.54 \pm 2.11) \times 10^{-6}$	$(5.30 \pm 0.05) \times 10^{-6}$

Table 2. Observed rate constants of the degradation of crystalline form of cefuroxime axetil in the presence of excipients.

Excipient	Condition of degradation	
	RH = 0%, T = 383 K $(k \pm \Delta k)$ [s^{-1}]	RH ~ 76%, T = 353 K $(k \pm \Delta k)$ [s^{-1}]
Aerosil	$(1.48 \pm 0.03) \times 10^{-6}$	$(4.74 \pm 0.05) \times 10^{-6}$
Mannitol	$(1.78 \pm 0.03) \times 10^{-6}$	$(1.85 \pm 3.21) \times 10^{-6}$
Crospovidone	$(2.02 \pm 0.02) \times 10^{-6}$	$(4.39 \pm 0.07) \times 10^{-6}$
Magnesium stearate	$(1.79 \pm 0.09) \times 10^{-6}$	$(1.64 \pm 0.01) \times 10^{-6}$
Microcrystalline cellulose	$(1.67 \pm 0.04) \times 10^{-6}$	$(2.89 \pm 0.11) \times 10^{-6}$
Croscarmellose	$(1.14 \pm 0.01) \times 10^{-6}$	$(3.82 \pm 0.45) \times 10^{-6}$

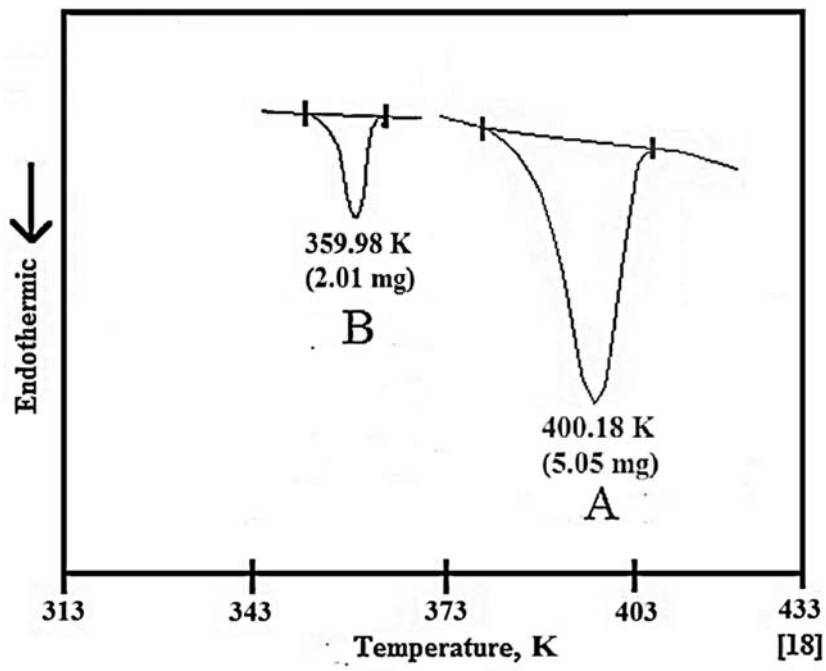

Figure 2. DSC analysis of crystalline form (A) and amorphous form (B) of cefuroxime axetil

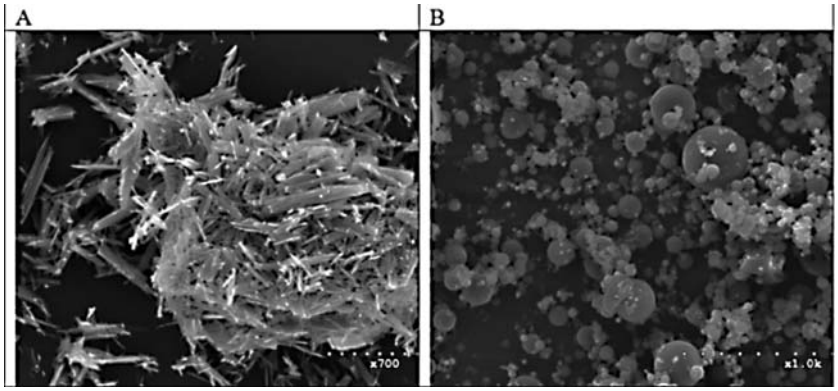

Figure 3. SEM photos of crystalline form (A) and amorphous form (B) of cefuroxime axetil

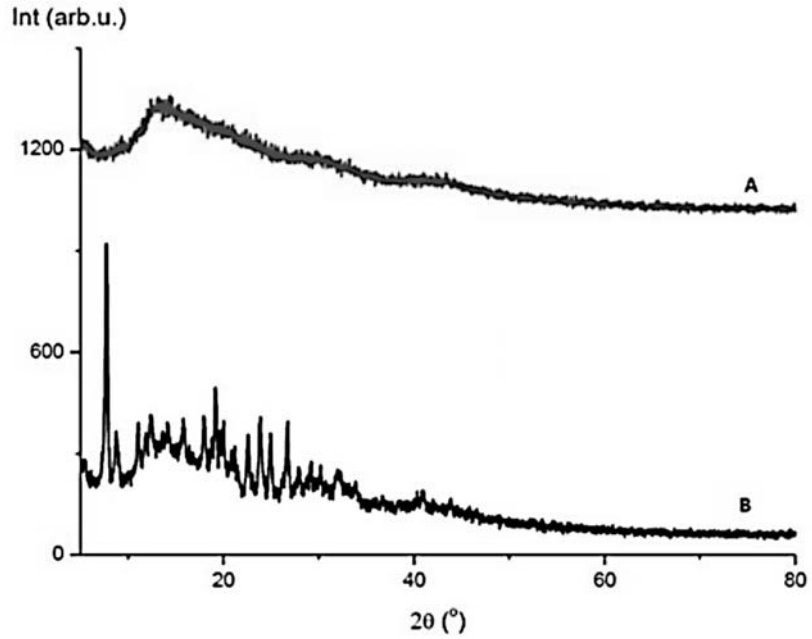

Figure 4. XRPD spectra of amorphous form (A) and crystalline form (B) of cefuroxime axetil

Figure 4. As expected, in the registered diffraction pattern of the crystalline phase diffraction peaks are clearly visible, which is a strong evidence of the crystalline character of the tested sample. In contrast to the crystalline phase, the diffraction pattern of amorphous CA shown only very broad and rather weak diffraction maxima, which usually are typical for amorphous samples. Only in 2θ angular range from 4 to 11° very weak diffraction peaks are observed, indicating a residual crystalline phase.

Compatibility studies of crystalline form of CA

The studies of the stability of crystalline and amorphous forms of CA in the presence of excipients were performed in dry air (RH = 0%, T = 383 K) and at increased relative humidity (RH ~ 76%, T = 353 K). Concentration changes of CA under the forced studies were evaluated by using the UHPLC-DAD method (19).

In the first stage of investigations, the stability of both polymorphic forms of CA was compared and as expected, the amorphous form exhibited greater susceptibility to degradation, under applied stress factors than crystalline form (Table 1).

Moreover, it was confirmed that the kinetic mechanism of degradation depends on the polymorphic form of CA. Amorphous form was degraded according to the first-order reaction model, depend-

ing on the substrate concentration in dry air while in increased humidity conditions proceeded according to the autocatalytic reaction model, relative to the substrate concentration, which were in accord with the results of previous studies (13, 14).

On the other hand, the decomposition of crystalline form of CA in dry air occurred according to the mechanism of reversible reaction and in increased relative humidity (RH ~ 76%) its degradation was autocatalytic reaction. As the effect of the impact of excipients on the stability of CA in crystalline form changes of the kinetic mechanism of degradation in comparison to the bulk substance were observed.

In dry air, during the degradation of crystalline form of CA, its concentration (C) decreased in the time interval $t_0 \rightarrow t_\infty$ from $(CCA)_{max}$ to $(CCA)_0$, therefore it was degraded according to the

Table 3. The summary of binding energies and HOMO - LUMO gaps of investigated systems of cefuroxime axetil - excipient.

Excipient	Binding energy [kJ/mol]	Relative binding energy	ΔHOMO - LUMO (eV)	Relative ΔHOMO - LUMO
Mannitol	-162.433	1.00	-8.57839	1.00
Croscarmellose	-312.664	1.93	-8.38927	0.98
Crospovidone	-218.377	1.34	-8.45349	0.98
Microcrystalline cellulose	-204.817	1.26	-8.12587	0.95

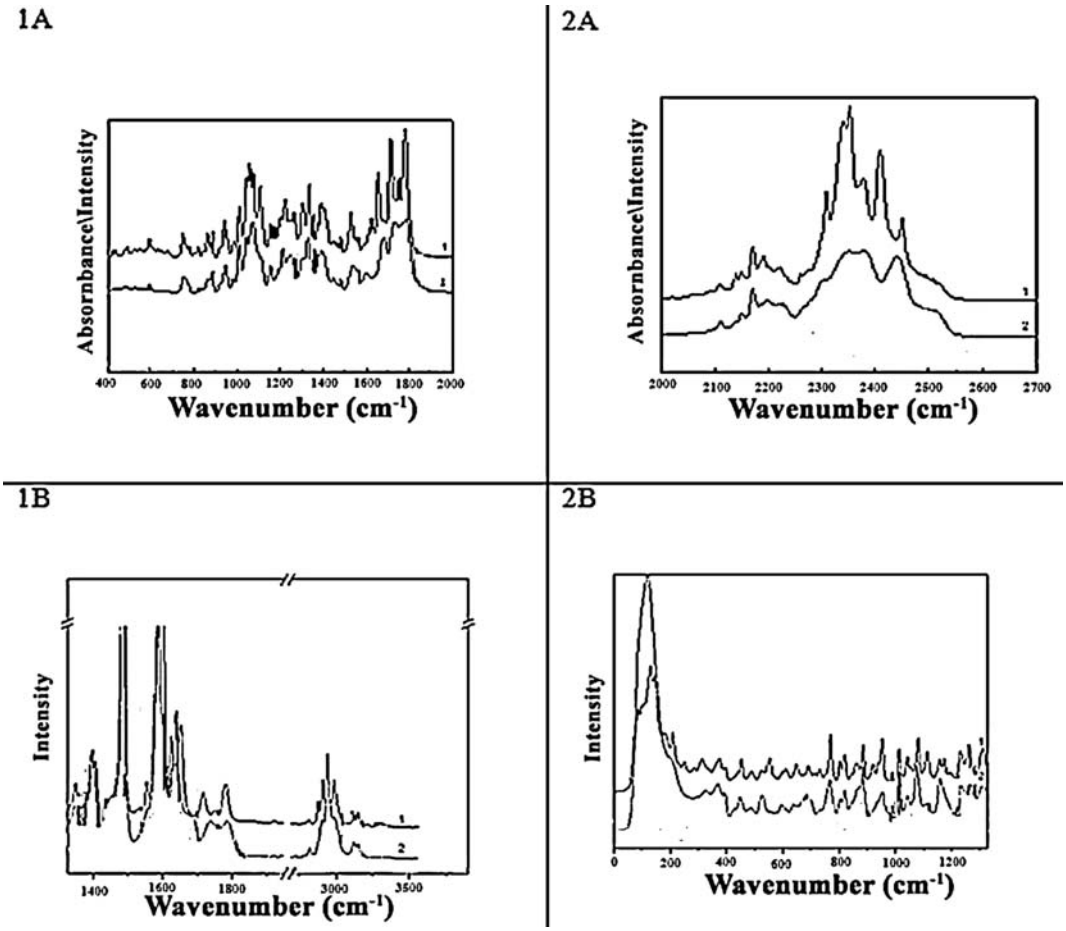

Figure 5. FT-IR absorption spectra of crystalline form (1A) and amorphous form (2A) of cefuroxime axetil and Raman specta of crystalline form (1B) and amorphous form (2B) of cefuroxime axetil at room temperature, in polycrystalline powder: (A) - crystalline form, (B) - amorphous form

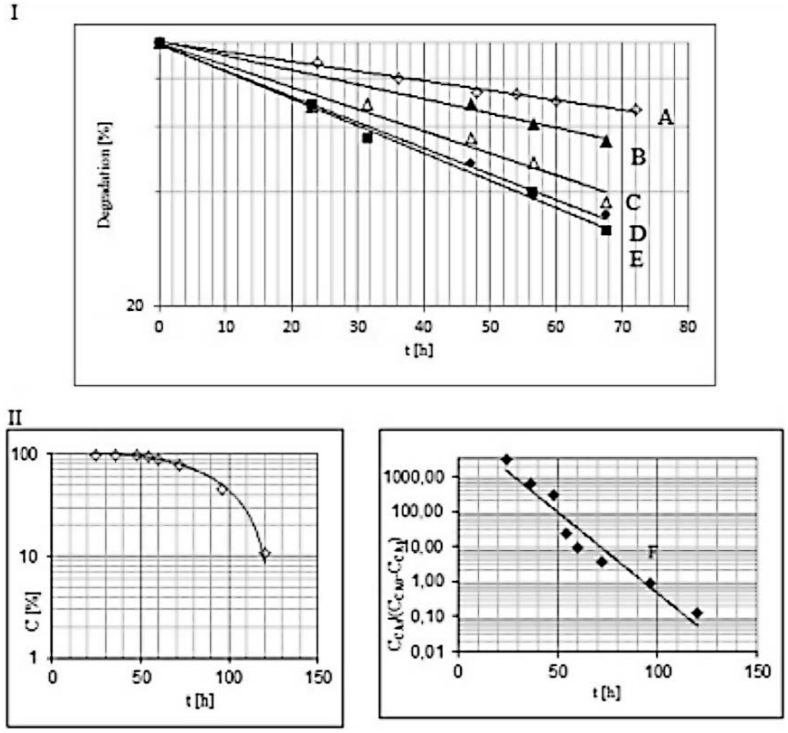

Figure 6. Semilogarithmic plots of CCA(%) = c (f) (I) and CCAt/(CCA0-CCAt)(%) = f(t) (II) in the presence of magnesium stearate (A), microcrystalline cellulose (B), crospovidone (C), aerosil (D), croscarmellose sodium (E) and mannitol (F) for the degradation of crystalline form of cefuroxime axetil at ~76% RH in solid state at 353 K where C_{CA} is the concentration of cefuroxime axetil, CC_{AO} is the concentration of cefuroxime axetil at 0 h and CC_{At} is the concentration of cefuroxime axetil at t h

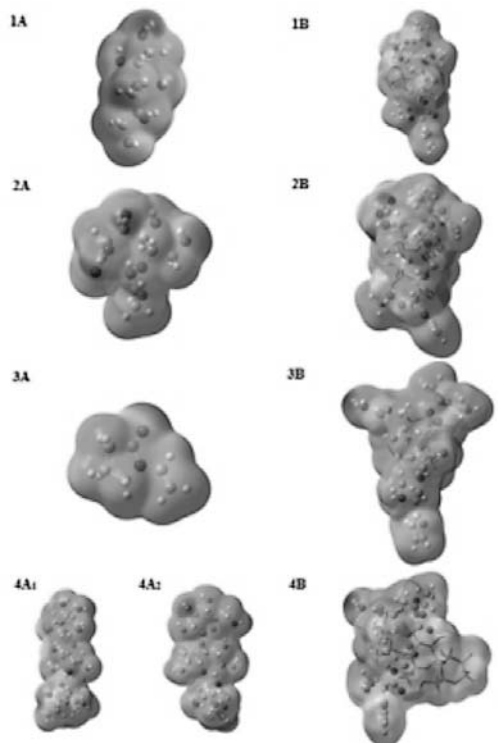

Figure 7. Electrostatic potential of excipient (A) and its complex with cefuroxime axetil (B); 1 - mannitol, 2 - croscarmellose, 3 - crospovidone, 4 - microcrystalline cellulose

first-order reaction, depending on the substrate concentration and may be described by the following equation:

$$\ln(CCA) = \ln(CCA)_0 - k_{obs} \cdot t \qquad (1)$$

The observed rate constants (k_{obs}) were equal to the slopes of the plots ln(PCA) = f(t) with a negative sign. In the case of all applied excipients, the kinetic mechanism of degradation under the applied conditions was the same. However, diastereoisomer A of crystalline form of CA exhibited greater lability than diastereoisomer B in its binary system with excipients. The observed rate constants describing the degradation of crystalline form of CA in the presence of selected excipients (except croscarmellose sodium) in the binary systems were slightly accelerated (Table 2).

Higher lability of CA in the presence of croscarmellose sodium can be explained by competition between weakly basic drug and the sodium counterion (20).

At the increased air humidity (RH~76%) and T = 353 K, the presence of aerosil, microcrystalline cellulose, magnesium stearate, croscarmellose sodium, crospovidone and magnesium stearate in binary systems appreciably changed the kinetic mechanism of degradation of crystalline form of CA from the

autocatalytic reaction to the first-order reaction model, depending on the substrate concentration, according to equation 1. The smallest, comparable values of observed rate constants of CA degradation were found in the presence of mannitol and magnesium stearate (Fig. 6). According to Waterman et al., different kinetic mechanism of degradation of CA in binary system could be a consequence of non-catalytic interactions between excipients and API molecules (21). The observed rate constants of CA, in binary mixtures with excipients are collected in Table 2. The kinetic mechanism of decomposition of CA in binary system with mannitol did not change compared to bulk substance. In system with mannitol CA was decomposed according the equation:

$$\frac{\ln(CCA)_t}{(CCA)_0 - (CCA)_t} = -k_{obs} \cdot t \qquad (2)$$

The changes in the concentration of CA were not linear because in this type of reaction an induction phase with a very small substrate loss is initially observed and it is followed by an acceleration phase that involves rapid degradation. The dependence: ln cDt/(cD0 - cDt) = f (t) was a straight-line relationship with a negative sign ($-k_{obs}$), which is presented in Figure 6. The comparison of curves describing autocatalytic degradation of CA alone and in the presence of excipient indicates the extended period of induction in binary systems. In our previous studies of the structures of degradation products after exposition of CA to increased relative humidity were detected (20). It is possible to suggest that interactions between formed degradation products and excipients eliminates their catalytic effect on CA degradation. In order to explain differences in interactions of excipients and cefuroxime axetil the theoretical approach was used. The analysis of ESP map of cefuroxime axetil showed (Fig. 1) that relatively high positive potential is located over the first-order amine group, while high negative charge is located over carbonyl group in cephem ring. These two potentials allow creation of API – excipient interaction. ESP map of optimized geometry of mannitol is presented (Fig. 7). Anti-symmetrically located electrostatic potential makes it difficult to fit mannitol with API compound. Despite that fact, AutoDock Vina docking software proposed most probable conformation of complex. ESP maps of croscarmellose and its complex were presented (Fig. 7). Croscarmellose has significant electric dipole moment, what results from oppositely placed electrostatic potentials of different signs. It allows inter-

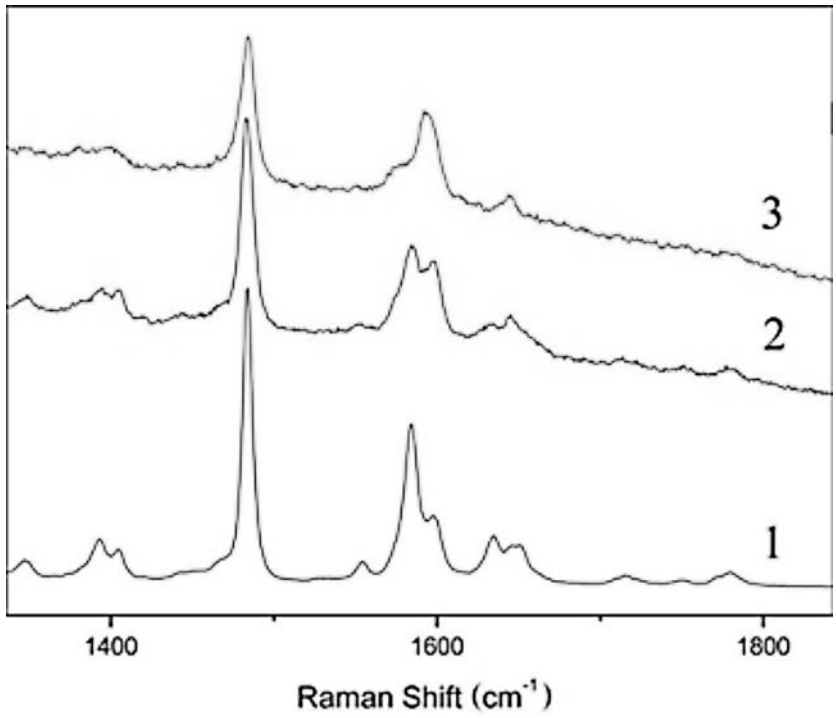

Figure 8. Raman spectra of cefuroxime axetil for non-degraded samples (1), degraded in dry air (RH = 0%, T = 383 K) (2) and degraded at an increased relative air humidity (RH ~ 76%, T = 353 K) (3)

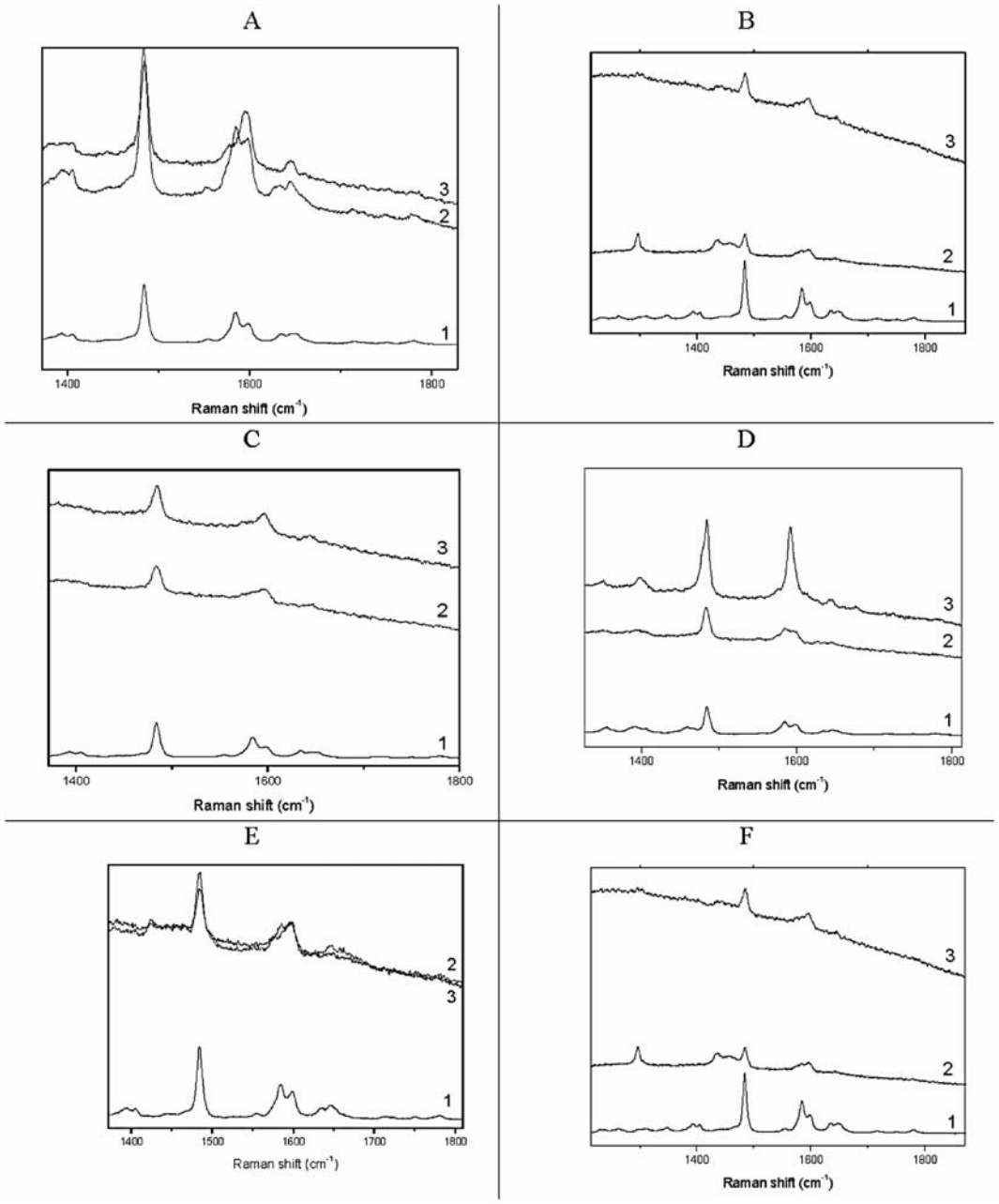

Figure 9. Raman spectra of cefuroxime axetil in the presence of microcrystalline cellulose (A), magnesium stearate (B), croscarmellose sodium (C), mannitol (D), crospovidone (E), and aerosil (F) for non-degraded samples (1), degraded in dry air (RH = 0%, T = 383 K) (2) and degraded at an increased relative air humidity (RH ~ 76%, T = 353 K) (3)

actions of modifier with mentioned first order amine group and carbonyl group of cefuroxime axetil. Crospovidone has significant positive charge over heterocyclic ring which seems to interact with carbonyl group in cefuroxime axetil. Celluose has positive charge located over glucose ring which interacts with carbon atom of carbonyl group, while oppositely located negative charge interacts with amine group of cefuroxime axetil, changing its geometry (Fig. 7). Described hypothetic complexes were further investigated. Complexes stability was computed in order to check if any significant interaction occurs between modifier and API, which can influence on degradation process. The interaction which is subject of investigation should explain changes in degradation curve of all API – excipient

systems, except API – mannitol system, where such change was not observed.

Energies of all modifiers and API were presented (Table 3).

Binding energy was computed according to equation 3.

$$E_{bind} = E_{API\text{-}Exc} - (E_{API} + E_{Exc}) \quad (3)$$

where $E_{API\text{-}Exc}$ is energy of API – excipient system, E_{API} and E_{Exc} are energies of free, isolated molecules of API and excipient, respectively. The computations were carried out with semiempirical method, thus it is recommended to compare relative energies of molecules in order to avoid systematic error of the method. The lowest binding energy belongs to API – mannitol system what stays in agreement with experiment and can explain no change in mannitol degradation curve. Additionally, calculations of HOMO – LUMO gaps were conducted to support stability studies described earlier. HOMO – LUMO energy gap is considered to be indicator of complex stability. The lower gap value, the less polarizable molecule is. Lowering HOMO – LUMO gap by addition of excipient results in higher stability of complexes and thus, lower reactivity. Computed gaps were presented in Table 3. The biggest frontier orbitals gap was noticed for API – mannitol system what confirms explicit studies of binding energies. According to ESP maps, computed binding energies and HOMO – LUMO gaps, difference between API - mannitol and other investigated systems degradation curves can be explained. Mannitol interactions were too weak to have considerable influence on degradation reaction, low binding energy gives low probability for API - mannitol system to appear in bound state.

Polymorphic transformation of crystalline form of CA

A study of the polymorphic transformation of cefuroxime axetil was started by defining differences between the character, position and intensity of bands in Raman spectra for the amorphous and crystalline forms of cefuroxime axetil. It was theoretically supported by the density functional theory. The most meaningful differences in the crystalline form spectrum were bands in the range 1504–1680 cm^{-1} (dublet), at 1635 cm^{-1} and at 1650 cm^{-1} (dublet), whereas in the spectrum of the amorphous form of the compound the differing bands were at 1593 cm^{-1} and 1636 cm^{-1}. The differences in the occurrence of those bands were an indication of the polymorphic transformation of the crystalline form of cefuroxime axetil. Since the most common causes of polymorphic transformations of active pharmaceutical substances include temperature, humidity and the co-presence of excipients, the study was also concerned with changes in the spectra of the crystalline form of cefuroxime axetil after its exposure to those factors and upon its inclusion in binary systems with such excipients as mannitol, microcrystalline cellulose, magnesium stearate, croscarmellose sodium, aerosil and crospovidone.

A polymorphic transformation towards the amorphous form was observed for samples of the crystalline form of cefuroxime axetil following its exposure to increased temperature in dry air (383 K) and in air with increased relative humidity (353 K, RH = 76%) (Fig. 8).

An analysis of the Raman spectra of mixtures of cefuroxime axetil with microcrystalline cellulose, magnesium stearate and croscarmellose sodium did not show a remarkably stronger tendency to transformation towards the amorphous form, in contrast to binary systems with mannitol and crospovidone. A comparable intensity of the bands at 1584 and 1598 cm^{-1}, characteristic of the amorphous form, was seen in the Raman spectra of the systems, whereas the bands of greater intensity diminished at 1584 cm^{-1} relative to the band at 1598 cm^{-1}. An examination of the Raman spectra of the crystalline form of cefuroxime axetil in binary systems with the excipients exposed to elevated temperature (383 K) in dry air for 24 h did not demonstrate a greater tendency to transformation towards the amorphous form or the co-presence of both forms. The crystalline form of cefuroxime axetil transformed to the amorphous form after exposure to increased temperature and humidity (353 K, RH = 76%) for 48 h in all binary systems with the excipients except aerosil, where the amorphous and crystalline forms were found coexistent (Fig. 9).

CONCLUSIONS

Analytical techniques based on DSC, SEM, XRD, FT-IR and Raman spectroscopy allowed a satisfying characterization of the amorphous and crystalline forms of cefuroxime axetil. A study of the compatibility of the crystalline form of cefuroxime axetil with excipients demonstrated their prominent impact on the kinetic mechanism and the rate of degradation. This was probably a result of the interactions between cefuroxime axetil and the excipients, for example the stabilizing effect of magnesium stearate, or degradation products, for instance the elimination of their catalytic effect on the degradation of cefuroxime axetil in binary systems after exposure to increased temperature (383 K). It was

found that the polymorphic transformation of the crystalline form of cefuroxime axetil to the amorphous form was possible as a consequence of its exposure to elevated temperature and/or increased relative air humidity. Under these conditions, the copresence of both polymorphic forms occurred only in a mixture of cefuroxime axetil with aerosil. In the context of monitoring the polymorphic transformation of the crystalline form of cefuroxime axetil in bulk substance and in systems with excipients, Raman spectroscopy may be recommended as a particularly valuable analytical technique that meets the criteria of being non-destructive, adequately rapid, ecological and able to analyze active pharmaceutical ingredients in the presence of water.

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FORMULATION AND *IN VITRO* EVALUATION OF ACYCLOVIR LOADED POLYMERIC MICROPARTICLES: A SOLUBILITY ENHANCEMENT STUDY

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Abstract: Objective of present work was to formulate polymeric microparticles of acyclovir using β-cyclodextrin by solvent evaporation method and kneading technique. Four different ratios were fabricated in each case. Sodium lauryl sulfate (4%) was utilized as intestinal permeation enhancer in this study. Prepared microparticles were characterized for micromeritic properties i.e., angle of repose, Hausner's ratio, Carr's index, bulk density and tapped density, entrapment efficiency, zeta size and zeta potential, Fourier transform infrared spectroscopy, differential scanning calorimetry, powder x-ray diffraction, scanning electron microscopy, transmission electron microscopy, optical microscopy and permeability studies across chicken intestine. Kinetic models: zero order, first order, Higuchi and Korsmeyer Peppas were applied on release data. Based upon the results of entrapment efficiency (81.25% and 74.50%), product yield (92.50% and 85.50%), permeability (85.18% and 82.05%), x-ray diffraction (amorphous nature), and solubility etc., (1 : 2) drug-polymer ratio was declared the best. Moreover, solid dispersions (1 : 2) had shown promising results. A new potential approach for solubility, bioavailability and permeability enhancement of acyclovir and other BCS class IV drugs was successfully established.

Keywords: acyclovir, solubility, permeability, β-cyclodextrin, solvent evaporation method, kneading technique.

To achieve therapeutic goals oral route still remains a preferable approach due to its simplicity, ease of administration, accurate and precise dosing. A majority of old chemical moieties i.e., 25–40% and 90% of newly discovered drugs exhibit inappropriate solubility and variable absorption across natural barriers that ultimately results in poor bioavailability of active pharmaceutical ingredient (API). Bioavailability of drugs with poor water solubility within the gastric mucosa can be enhanced by enhancing solubility of drug using different approaches (1). Among them, one of the approaches is altering nature of the drug i.e., from crystalline to amorphous form. Bioavailability of water insoluble drugs increases by increasing dissolved fraction consequently improving systemic availability of APIs (2).

Acyclovir [9-(2-hydroxyethoxy)-methyl]-guanine, is a drug of choice against herpes simplex virus (HSV), varicella zoster virus (VZV), Epstein bar virus (EBV), cytomegalovirus (CMV) and human herpes virus (HHV-6). It is activated by viral thymidine kinase enzyme into tri-phosphate form and inhibits viral DNA polymerase enzyme to stop viral replication. Acyclovir has oral bioavailability of only 10-30% due to its poor solubility (2.5 mg/mL, 37 ± 2°C) and low permeability. It is an ampholyte drug with two pKa values and is completely soluble at pH 1.2 and pH 7.4. Plasma half-life is about 2.5 h. So frequent dosing i.e., 200 mg five times a day is required to attain desired drug concentration within therapeutic levels. It has no fixed place in Biopharmaceutics Classification System (BCS) i.e., at 200 mg it is placed in BCS-III and at dose of 800 mg, it show properties of BCS-IV drugs (2, 3).

Drug polymeric complexes in the form of microparticles can be formulated by utilizing water soluble and water insoluble polymers of synthetic, semisynthetic and natural origin. Cyclodextrins (CD) are circular oligosaccharides containing 6-8 glucose units that are connected with each other by α-1,4 glucosidic linkages. CD's are abundantly used for solubility, dissolution, stability and bioavailability enhancement. Among CD's, β-cyclodextrin (β-CD) has major share in different fields because of low

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price, availability and biocompatibility. It presents itself as amphiphilic moiety i.e., hydrophilic outer surface due to presence of (–OH) groups and hydrophobic inner cone with less polarity. It has ability to grab therapeutic drug molecules with molecular weight 200 – 800 g/mol. β-CD is widely accepted as solubility, stability and bioavailability enhancing agent in the form of micro-sized particles i.e., microparticles (solid dispersions and inclusion complexes) (4).

A bunch of approaches has been presented in the literature to improve solubility and bioavailability of poorly soluble drugs. These include use of co-solvents, surfactants, cyclodextrins, salt formation, rapid dissolving tablets, pH maintenance, particle size reduction, hydrogel microparticles and lipids based delivery systems (5). Particles with micrometric size (microparticles) range can be used as potential candidates for solubility issues of hydrophobic drugs due to large surface area, wettability and wicking properties.

Moreover, permeability issues can be minimized by the addition of surfactants, chelating agents, fatty acids and cyclodextrins etc. Anionic surfactants i.e., sodium lauryl sulfate (SLS also called as sodium dodecyl sulfate) have an efficient wetting, solubility, dissolution, stability and permeability enhancement characteristics when these are available in nearby locality of dissolving products.

Solid dispersion (SD) is the dispersion of drug molecules into a non-reactive polymeric carrier by different approaches like melting, co-grinding, use of solvent and size reduction etc. Due to amorphous nature of SD's these provide enhanced dissolution, increased surface area, improved wettability of active drug and inhibit recrystallization in physiological fluids of the body (6).

On the other hand, inclusion complexes host forms cone/cavity in which second molecule resides forming an inclusion complex. There stable interaction is strongly supported by Van der Waals forces, hydrogen boding, electrostatic forces, hydrophobic interactions and charge transfer interaction (7).

In present work, efforts have been made to improve solubility, dissolution and bioavailability of acyclovir by developing β-CD and surfactant based polymeric microparticles i.e., SD's and inclusion complexes (IC's) with desired characteristics of solubility, dissolution and bioavailability.

EXPERIMENTAL

Materials

Chemicals utilized in this study included: acyclovir received as gift sample from Brooks Pharmaceuticals, Karachi, Pakistan, β-cyclodextrin 97% pure purchased from Sigma Aldrich, Germany, sodium lauryl sulfate obtained from Gray's Pharmaceuticals, Islamabad, Pakistan. Methanol and hydrochloric acid of HPLC grade were purchased from Merck, USA. Double distilled water was freshly prepared by LC/MS Lab no. 25 of department. All the chemicals and solvents used were of analytical grade.

METHODS

Preparation of acyclovir – β-cyclodextrin solid dispersions

Acyclovir loaded β-cyclodextrin solid dispersions were obtained by modifying solvent evaporation method used by Frizon et al. (8). Five different ratios (drug and polymer) were selected i.e., 1 : 0.5, 1 : 1, 1 : 2, 1 : 3 and 1 : 4. Required quantities of β-CD, acyclovir (ACV) and sodium lauryl sulfate (1, 2, 4 and 6%) were weighed on electronic weighing balance (Shimadzu, AUW220D Japan). β-CD and sodium lauryl sulfate were poured into beaker containing water-methanol mixture (25 : 75, v/v) that was already placed on hot plate magnetic stirrer (30°C) at 100 rpm. Stirring was continued until clear solution was formed. Acyclovir 1% solution was prepared by pre-dissolving ACV in 0.1 mol/L HCl media and sonicated to completely solubilize it. Acyclovir solution was then poured into β-CD solution and stirring was continued at 100 rpm and 40°C until the whole solvent was evaporated. End product was spread on filter paper and placed in hot air oven (Memmert) at 50°C for complete drying overnight. Dried solid dispersions were sieved through sieve no. 80 to attain uniform particle size distribution. Formulations prepared by this method were tagged as SD1 (1 : 0.5), SD2 (1 : 1), SD3 (1 : 2), SD4 (1 : 3) and SD5 (1 :4), respectively, and stored in air tight containers containing silica gel bags for further analysis (8).

Preparation of acyclovir – β-cyclodextrin inclusion complexes

Acyclovir loaded β-CD inclusion complexes were fabricated by kneading technique. Four different (drug-polymer) ratios were designed i.e., 1 : 0.5, 1 : 1, 1 : 2, 1 : 3 and 1 : 4. β-CD and sodium lauryl sulfate were accurately weighed and subjected to pestle and mortar by adding water-methanol (25 : 75) mixture dropwise to form paste. Acyclovir 1% solution was prepared in 0.1 mol/L HCl media as discussed above. SLS was used in 1, 2, 4 and 6% in

all four ratios, respectively. Dissolved ACV drug solution was added into β-CD, sodium lauryl sulfate paste and trituration was further continued for 3 h. Mortar was then placed into hot air oven (Memmert) at 50°C overnight for complete drying of the product. Fabricated product was sieved through sieve no. 80 to obtain particles of uniform size. Inclusion complexes thus formed were named as IC1 (1 : 0.5), IC2 (1 : 1), IC3 (1 : 2), IC4 (1 : 3) and IC5 (1 : 4). They were stored in air tight containers for further experimental work (9).

Solubility studies

Solubility studies were performed at pH 1.2 and pH 7.4. An excess amount of acyclovir – β-CD complex was poured into 1 mL of different solutions i.e., pH 1.2, pH 6.8 and water in separate screw capped glass vials. All solutions were subjected to mechanical shaking on thermo shaker incubator (MSC-100 China) operated at 400 rpm and 37 ± 5°C for 24 h to attain equilibrium. Each solution was then subjected to centrifugation at 6000 rpm for 10 min. Supernatant was collected by micropipette and filtered through 0.45 µm syringe filter (Sartorius). One mL of filtered solution was further diluted by distilled water and marked a dilution number accordingly. Each dilution was analyzed at 254 nm using UV-visible spectrophotometer (Shimadzu, Japan) against the same β-CD concentration in water for omission of any absorbance from polymer (10).

Product yield

Actual amount of ingredients incorporated into microparticles and amount of microparticles recovered at the end of each preparation method was noted (11). Product yield in percentage was calculated using following formulas:

$$Mass\ loss\ (\%) = \frac{M_0 - M_1}{M_0} \times 100$$

$$Product\ yield = 100 - mass\ loss$$

where M_o = initial weight of all ingredients, M_1 = final weight of microparticles.

Entrapment efficiency

Microparticles containing ACV were weighed on electronic weighing balance (Shimadzu, AUW 220D, Japan), triturated, dissolved in phosphate buffer pH 7.4 and filtered. After filtration, aliquots were further diluted and absorbance was determined by using UV-visible spectrophotometer at 254 nm (11). The amount of acyclovir incorporated into microparticles was determined using the following equation:

$$Entrapment\ efficiency = \frac{Absorbance\ of\ sample\ containing\ 50\ mg\ ACV}{Absorbance\ of\ 50\ mg\ pure\ ACV} \times 100$$

Micromeritic properties

Angle of repose

Funnel method was used to calculate angle of repose. Prepared microparticle's blend was poured through vertically placed funnel having aluminium sheet under it. Height (h) of cone was noted by scale and radius (r) was calculated by drawing the diameter of cone by pencil on aluminium sheet and then dividing it by 2. Angle of repose (θ) was calculated using the following formula:

$$Tan\ \theta = \frac{h}{r}$$

Angle of repose less than 30° supports better flow properties of prepared microparticles.

Bulk density

Cylinder method was adopted for bulk density. Microparticles of known mass (M) were taken. These were poured into measuring cylinder and their bulk volume (Vb) was noted. Bulk density (*ńb*) was calculated using the following formula:

$$Bulk\ density\ (\rho b) = \frac{M}{Vb}$$

Tapped density

Cylinder method along with tapings i.e., 250 ± 15 taps from a height of 3 ± 0.2 mm was adopted for tapped density. Known mass was added into measuring cylinder and subjected for tapping. Tapped volume (Vt) was noted after tapping visually (12). Tapped density was calculated using the following formula:

$$Tapped\ density\ (\rho t) = \frac{M}{Vt}$$

Carr's compressibility index

Carr's index (I) was calculated by using results of tapped volume (Vt) and bulk volume (Vb) from their respective densities. It was calculated using the following formula:

$$Compressibility\ index\ (I) = \frac{Vt - Vb}{Vb} \times 100$$

Carr's index between 13 – 19% proves better flow of microparticles (12).

Hausner ratio

Ratio between tapped (ρt) and bulk (ρb) densities is known as Hausner ratio. The following formula was used for its calculation:

$$Hausner\ ratio = \frac{\rho t}{\rho b}$$

Value less than 1.25 provide evidence for good flow (12).

Instrumental analysis

Particle size and zeta potential determination

Particle size and zeta potential determination was made by single particle size analyzer (Zetasizer Nano-series ZEN3600, Malvern Instruments Ltd., U.K.) with software DTS-nano (8).

Microscopic analysis

Size analysis of prepared microparticles was performed using optical microscope (Nikon E200, Tokyo, Japan) equipped with (DCM-35 USB 2.0 and MINISEE IMAGE software, Scopetek Electric, Hangzhou, China). Samples were of white color so they were first soaked into black ink and dried on filter paper. Dried microparticles were spread and transparent tap was wrapped to fix particles on glass slide. Magnification power was set at 1009 (13).

Fourier transform infrared spectroscopy (FT-IR) analysis

FT-IR spectra of acyclovir, β-CD and prepared microparticles were recorded to check compatibility, complex formation or any sort of interaction by using FT-IR spectrophotometer (Tensor 27, Bruker, OPUS software). Samples were placed on crystal spot (zinc selenide) and arm was rotated downwards to generate fine disc of sample. Scanning range was kept within the range of 3500 to 1000 cm^{-1} at a spectral resolution 4 cm^{-1} (8).

Thermal analysis (DSC and TGA)

In order to get the phase transition and weight loss preview, acyclovir, β- CD, sodium lauryl sulfate and fabricated microparticles i.e., inclusion complexes and solid dispersions were examined using simultaneous thermogravimetric analyzer and differential scanning calorimeter (TA Instruments, USA model Q600). Before loading sample, both reference pan and sample pan were tarred. Samples were prepared after trituration and sealing in aluminium pan. Heating rate was kept 10°C/min for 0 to 400°C. Thermal stability of microparticles was determined by thermal gravimetric analysis (TGA). In both cases nitrogen flow rate was kept 10 mL/min (8).

Powder X-ray diffraction analysis (PXRD)

PXRD was carried out to check the impact of both processes on crystallinity of the acyclovir. PXRD patterns using XRD xpert pro with software pan analytical®. Voltage applied was 38 kV with current of 26 mA and scanning was performed at 2θ range 15-65°. Microparticles were triturated before taking each scan (8).

Scanning electron microscopy (SEM)

Surface morphology of prepared microparticles was observed on electron microscope (Quanta 250) using maker fei® software. Accelerating current was kept at 20 kV. Powder of microparticles was fixed on support with carbon-glue and coated with gold using a SPI sputter module in a high-vacuum evaporator (8).

Transmission electron microscopy (TEM)

More deep insight on prepared microparticles was taken by using JEM 2100F field-emission transmission electron microscope (JEOL, Tokyo, Japan). Samples were observed after sprinkling on metallic grid of instrument and different magnifications were used to obtained fine view (14).

Dissolution profile

Drug release studies were carried out in 0.1 M HCl of pH 1.2 and phosphate buffer of pH 7.4. Weighed quantity of prepared microparticles was enclosed in empty hard gelatin capsules against Cap. Zaclovir. Proper sink conditions were maintained. USP type-II apparatus was used. It was operated at 50 rpm at 37 ± 5°C. Five mL of samples were taken from middle of basket by using 10 mL pipette (Pyrex®) at predetermined time intervals and replaced with fresh dissolution media each time. Samples were filtered through syringe filter (5 µm pore), diluted and analyzed on UV-VIS Spectrophotometer (Pharma Spec 1700, Shimadzu, Japan) at 254 nm wavelength. All measurements were made in triplicate (8).

Permeability assessment using chicken intestine

Composition of plasma membrane of intestinal epithelium is similar among many species. Chicken intestine was selected for permeability studies due to its ease of availability and very low cost. Developed method of Dias and co-authors (15) with slight modifications was used. Intestinal part (small intestine) of white female chicken (broiler) was obtained from nearby market and shifted to lab in sealed polythene bag. Sterile sharp edge surgical blade of carbon steel was used to cut a segment of 6 cm length. Eversion of intestinal segment was made by mounting and rolling back on glass rod. Isotonic solution (Macsol NS) was used for rinsing and removing mucous and debris from outer absorptive. One end was closed tightly with silk thread and 25-30 mL of phosphate buffer solution (pH 7.4) was poured as dissolution media for microparticles. Other end of intestine was also tied making it as intestinal pouch and hanged in centre of dissolution media with paddle of dissolution appara-

tus. Prepared microparticles were enclosed in hard gelatin capsules and placed in the basket of dissolution apparatus. Temperature was maintained by thermostat at 37 ± 2°C. Apparatus was operated at a speed of 20 rpm. Sample (2 mL) was withdrawn from pouch using 3 mL disposable syringe. Furthermore, it was filtered, diluted and analyzed at 254 nm. Results were calculated in triplicate (15).

Stability studies

Stability studies were carried out on optimized formulation SD3 and IC3 according to ICH (International Conference on Harmonization) guidelines for a period of 3 months at 40 ± 2°C/75% RH ± 5% conditions in stability chamber (Memmert Beschickung, 100–800, Japan).

RESULTS

Solubility studies

Acyclovir is an ampholyte having pKa values 2.27 and 9.25. It has solubility equal to 0.005 mM/L in water. It is more soluble at pH 1.2 as compared to pH 7.4. Higher solubility was seen at lower pH of 1.2 than higher pH values. From solubility studies of ACV and microparticles, i.e., IC's and SD's, there was significant increase solubility of drug in water (7.6 fold), pH 1.2 (9.5 fold) and pH 7.4 (6 fold) as shown in Figure 1.

Entrapment efficiency and product yield

Results of entrapment efficiency and product yield are shown in Table 1. Results indicated that

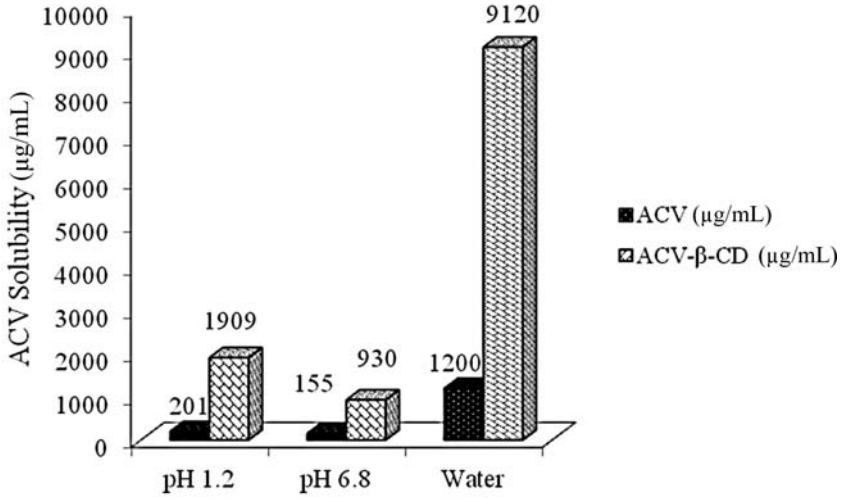


Figure 1. Column graph of solubility of ACV

Table 1. Results of entrapment efficiency and product yield.

Codes	Entrapment efficiency (%)	Product yield (%)
SD1 (1 : 0.5)	53.25 ± 0.25	67.00 ± 0.40
SD2 (1 : 1)	59.14 ± 0.15	80.00 ± 0.30
SD3 (1 : 2)	81.25 ± 0.50	92.50 ± 0.70
SD4 (1 : 3)	75.50 ± 0.25	91.50 ± 0.10
SD5 (1 : 4)	73.40 ± 0.85	91.75 ± 0.25
IC1 (1 : 0.5)	49.50 ± 0.10	64.00 ± 0.50
IC2 (1 : 1)	57.20 ± 0.30	78.50 ± 0.25
IC3 (1 : 2)	78.50 ± 0.25	85.50 ± 0.15
IC4 (1 : 3)	72.20 ± 0.50	86.20 ± 1.00
IC5 (1 : 4)	70.00 ± 0.25	86.80 ± 0.50

both of these parameters were ranged between 53.25 to 81.25% and 49.50 to 78.50% for SD's and IC's. respectively (Table 1). Optimum entrapment efficiency and product yield were observed for SD3 and IC3 formulations, i.e., 81.25 and 78.50% and 92.50 and 86.80%, respectively.

Micromeritic properties

In case of solid dispersions, angle of repose was found between 23.21° and 28.21° while in case of inclusion complexes it was ranged from 25.21° to 28.21° indicating overall better flow of powder blend. Bulk density of all microparticles formulations were ranged between 0.621-0.658 g/mL. Tapped density was calculated and found between 0.738-0.771 g/mL. Hausner ratio was found within 1.16 to 1.19 for all developed formulations. Similarly, Carr's index values were noted in the range of 16.03 to 19%. Individual results are presented in Table 2.

Particle size and zeta potential determination

There was no net charge on fabricated microparticles thereby confirming their stability. All

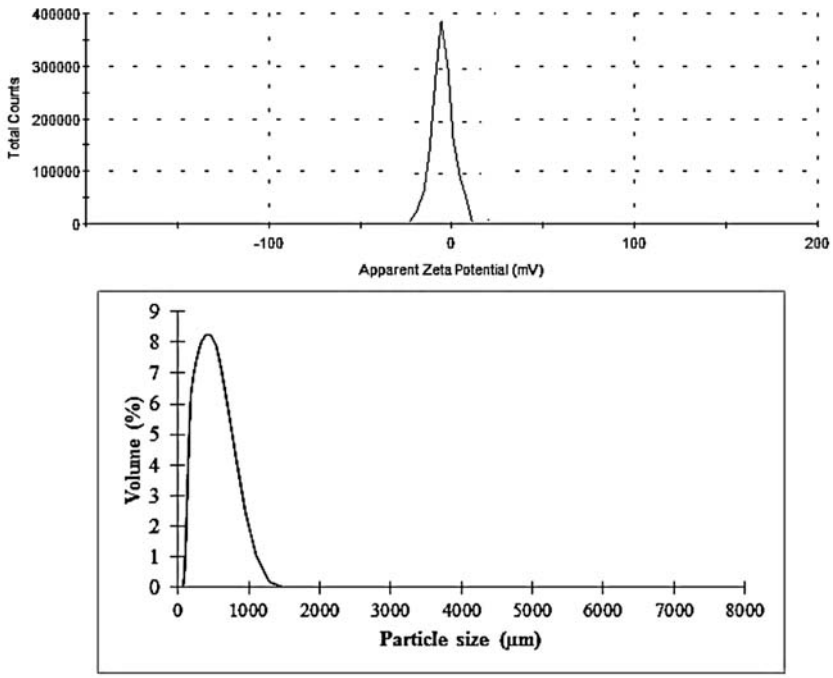


Figure 2. Zeta potential and particle size of microparticles

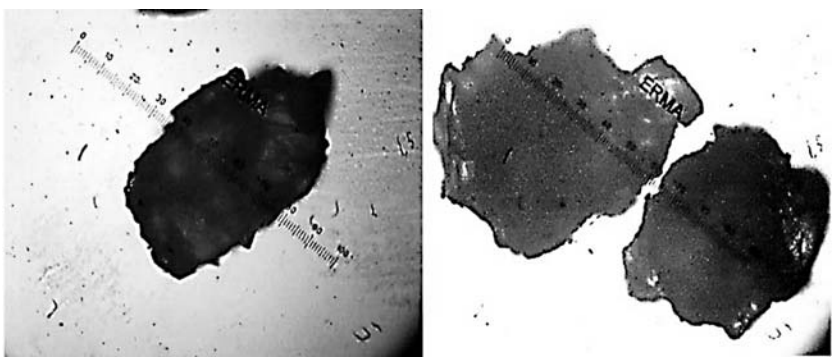


Figure 3. Photomicrograph of SD3

Table 2. Results of angle of repose, bulk density, tapped density, Hausner's ratio and Carr's index.

Code	Angle of repose (^{o})	Bulk density (g/mL)	Tapped density (g/mL)	Hausner's ratio	Carr's index (%)
SD1	23.21 ± 0.769	0.621 ± 0.005	0.739 ± 0.012	1.19 ± 0.012	19.00 ± 0.772
SD2	26.21 ± 0.991	0.632 ± 0.002	0.738 ± 0.011	1.16 ± 0.005	16.77 ± 0.844
SD3	24.21 ± 0.811	0.637 ± 0.001	0.757 ± 0.002	1.18 ± 0.001	18.83 ± 0.922
SD4	28.21 ± 0.521	0.661 ± 0.001	0.767 ± 0.004	1.16 ± 0.002	16.03 ± 0.976
SD5	25.80 ± 0.432	0.665 ± 0.003	0.782 ± 0.015	1.17 ± 0.003	14.96 ± 0.784
IC1	27.21 ± 0.724	0.639 ± 0.002	0.758 ± 0.004	1.18 ± 0.003	18.62 ± 0.916
IC2	28.21 ± 0.715	0.645 ± 0.003	0.749 ± 0.001	1.16 ± 0.001	16.12 ± 0.736
IC3	25.21 ± 0.803	0.642 ± 0.004	0.760 ± 0.003	1.18 ± 0.002	18.38 ± 0.806
IC4	28.21 ± 0.759	0.658 ± 0.003	0.771 ± 0.001	1.17 ± 0.001	17.17 ± 0.772
IC5	23.65 ± 0.673	0.672 ± 0.001	0.789 ± 0.003	1.17 ± 0.002	14.82 ± 0.638

S.D. (Standard deviation) ± Mean, n = 3.

Table 3. *In-vitro* drug release kinetics of acyclovir at pH 6.8

	Parameters	SD3	IC3	Cap. Zaclovir
Zero order	K_o	1.927	1.750	0.535
	R^2	0.9320	0.9642	0.9946
	T_{25}	12.972	14.285	46.704
	T_{50}	25.944	28.569	93.408
	T_{75}	38.916	42.854	140.112
First order	K_1	0.037	0.030	0.005
	R^2	0.9969	0.9975	0.9965
	T_{25}	7.880	9.556	53.265
	T_{50}	18.987	23.024	128.337
	T_{75}	37.974	46.049	256.674
Higuchi	K_H	11.759	10.795	3.095
	R^2	0.9970	0.9751	0.9077
Korsmeyer Peppas	K_{kp}	27.208	21.057	1.166
	R^2	0.9109	0.8930	0.9947
	n	0.254	0.300	0.764

the particulate complexes were having neutral value of charge on them. Microparticles were of micrometric scale having particle size in the range of 1–1000 µm as shown in Figure 2. Optical microscopic studies also proved micrometric range. Results are shown in Figure 3.

Fourier transform infrared spectroscopy

FT-IR spectra of each constituent as well as finished products i.e., solid dispersions and inclusion complexes were recorded. Results are shown in Figure 4. Acyclovir FTIR spectrum was very similar to be presented in the literature exhibiting characteristic peaks of primary and secondary amines at 3438 and 3179 cm^{-1}, one C=O band was seen at 1706 cm^{-1}, two bands at 1609 and 1629 cm^{-1} were of amino groups. O-H bands were recognized at 1541 and 1574 cm^{-1} and the presence of band at 1344.95 cm^{-1} confirmed –CH group of acyclovir.

IR spectrum of β-CD showed an evident wide absorption band due to vibrations of O-H bonds in primary C-OH groups at 3292 cm^{-1}, vibrations of C-H bonds hosted in -CH and $–CH_2$ groups presented absorption peak at 2927 cm^{-1} and due to vibrations of C-O bonds in ether and hydroxyl groups, peaks appeared at 1021 to 1077 cm^{-1} (16).

Thermal analysis (DSC and TGA)

DSC thermogram of β-CD has shown two endothermic peaks at 81.25 and 324.95°C corresponding to loss of moisture of crystallization and phase transition (solid to liquid), respectively. One exothermic peak was originated at 382.31°C, while in TGA curves of β-CD, three step mass losses were seen i.e., at 109.44°C and 3.95 min there was only 13.03% weight loss occurred, above melting point 326.86°C and 14. 86 min 18.35% mass loss occurred and at 359.21°C and 16.44 min mass loss observed was 84.28%. DSC of ACV has depicted less prominent peak at 90.88°C that relates to loss of moisture and broad endothermic peak at 256.80°C (with enthalpy variation of 0.05210 J/g) presenting its melting point. In TGA, prominent decomposition was observed at 268.69°C (Δm = 93.89%) (17). Results for fabricated IC's and SD's have been shown in Figure 5.

Powder X-ray diffraction analysis (PXRD)

ACV exists in the literature with six different crystal morphologies i.e., I, II, II, IV, V and VI. Nature of pure ACV powder, crystalline or amorphous was confirmed by PXRD studies. ACV has proved its crystalline nature due to prominent peaks at 2θ = 10.345°, 23.655°, 25.985°, 26.395° and 29.15°. Crystalline nature of CD was proved by the presence of sharp peaks at 2θ = 21.451°, 24.85°, 28.75°, 36.15° and 42.271° (17). All the results are shown in Figure 6.

Scanning electron microscopy (SEM)

Photomicrographs of IC's and SD's were scanned at various powers were taken to analyze

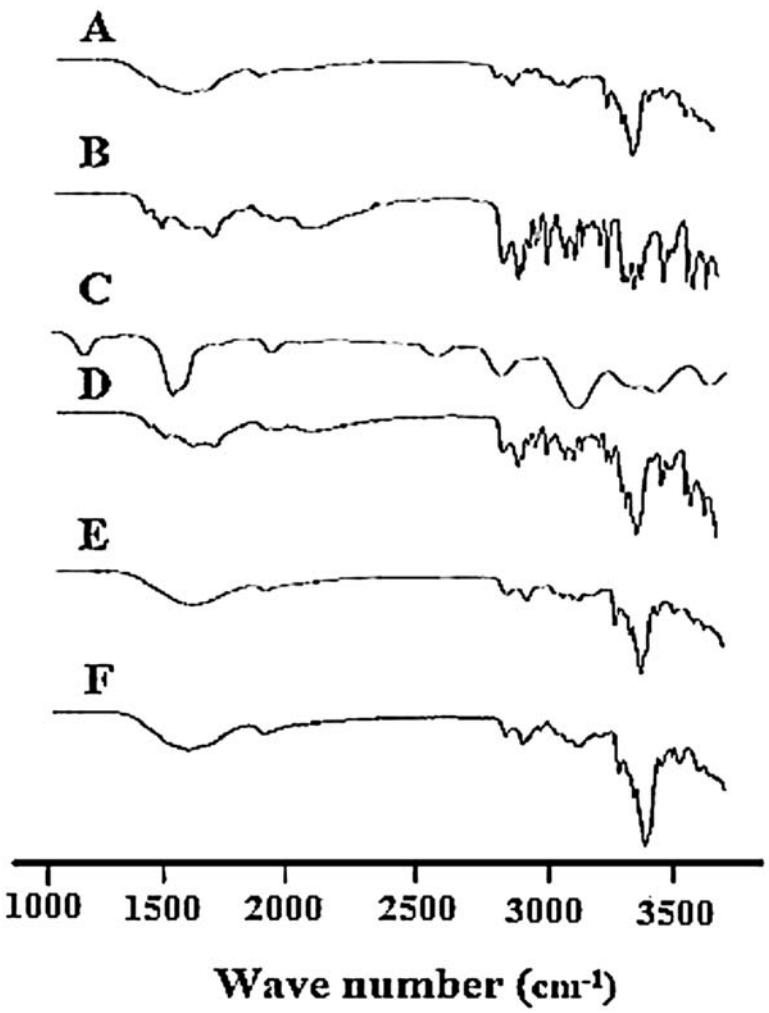


Figure 4. FTIR spectra of **A** - β-cyclodextrin, **B** - acyclovir, **C** - sodium lauryl sulfate, **D** - physical mixture, **E** - solid dispersions and **F** - inclusion complexes

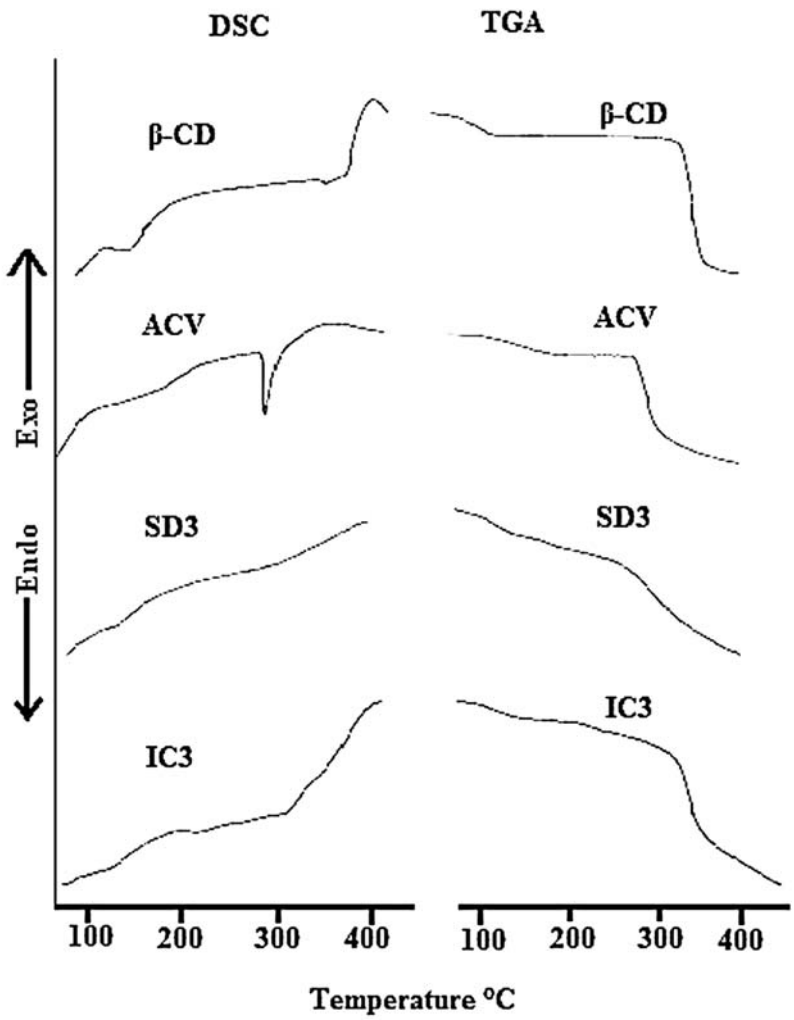


Figure 5. DSC and TGA thermograms

surface morphology. Irregular shape and size variant microparticles were seen but these were within pharmacopoeial limits of micrometric scale. All the results are presented in Figure 7.

Transmission electron microscopy (TEM)

Microparticles shape and more close morphology was seen in transmission electron microscopy images. Solid dispersions were dense carrying ACV with irregular shape but with definite boundaries, on the other hand, inclusion complexes were more uniform than solid dispersions. All results have been included in Figure 8.

Dissolution studies

Dissolution studies were conducted to asses release behavior at different pH values (pH 1.2 and 7.4). Slightly lower release profiles were seen at pH 7.4 as compared to pH 1.2 depending upon degree of ionization. SD's and IC's exhibited 90.75% and 93.12% at pH 1.2, respectively, while in case of ACV capsule drug release was only 59% during 3 h of study. At pH 7.4, 88.75% and 92.50% release was seen in case of inclusion complexes and solid dispersions, respectively. Results of best formulations i.e., IC3 and SD3 along with marketed product (Zaclovir) are shown in Figure 9. Drug release kinetic models i.e., zero order, first order, Higuchi and Korsmeyer Peppas were applied on data obtained up to maximum release period i.e., 60 min by using DD Solver® Excel based Add in program. Results are shown in Table 3.

Permeability studies

ACV is weakly permeable across intestinal mucosa. To overcome such issue, fixed quantity

(4%) of sodium lauryl sulfate was incorporated into each ratio of finished microparticles. Optimum percentage of diffused drug was noted in IC3 and SD3 i.e., 82.05% and 85.18%, while Zaclovir marketed capsule has only shown 36.89% diffusion. Results are presented in Figure 10.

Stability studies

No change in dissolution, permeability profile and physical morphology was observed after stability studies.

DISCUSSION

Solubility studies

At pH 1.2, reason for high solubility was that at this pH ACV exists in ionized form. Moreover, SLS incorporated for permeation enhancement has excellent wetting, solubility, dissolution and stability properties. It might have contributed for enhanced solubility of ACV with rising ratios of β-CD. Solubility studies revealed 1 : 1 complex formation in aqueous solution because of stability constant obtained from phase solubility diagram. Kumari et al. have made efforts for solubility enhancement of nelfinavir mesylate using different ratios of β-CD in the form of solid dispersions and inclusion complexes. At pH 1.2 there was 5.32-fold rise in solubility while at higher pH 3.64-fold rise was seen that supported solubility results of present study (18).

Entrapment efficiency and product yield

Entrapment efficiency of SD's and IC's was increased from 53.25% to 81.25% and 49.50% to 78.50%, respectively (1 : 0.5 to 1 : 2), after that a decline was noted in case of 1 : 3 and 1 : 4. Main reason behind this was that increasing polymeric content results in more compact or packed polymer coat that limit further entrapment of ACV. Product yield was 92.50%, 86.80% for SD3 and IC3. Study conducted by Tummala et al. exhibited higher entrapment of 5-flurouracil (69.18%) within polymeric network up to 1 : 3 drug-polymer ratio, then there

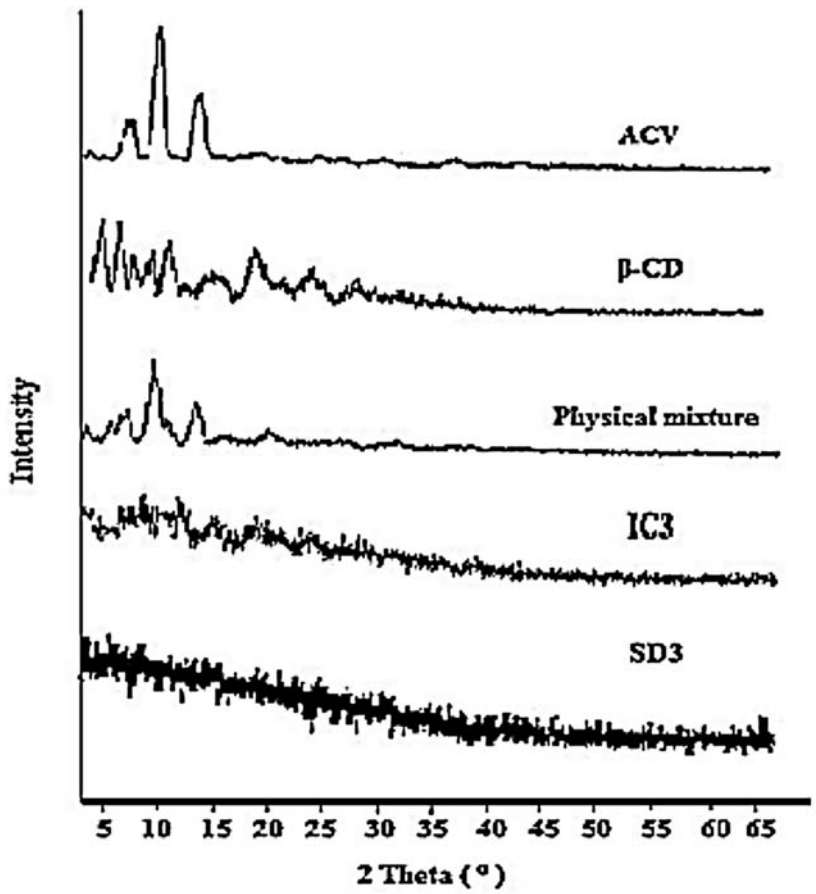


Figure 6. XRD diffractograms

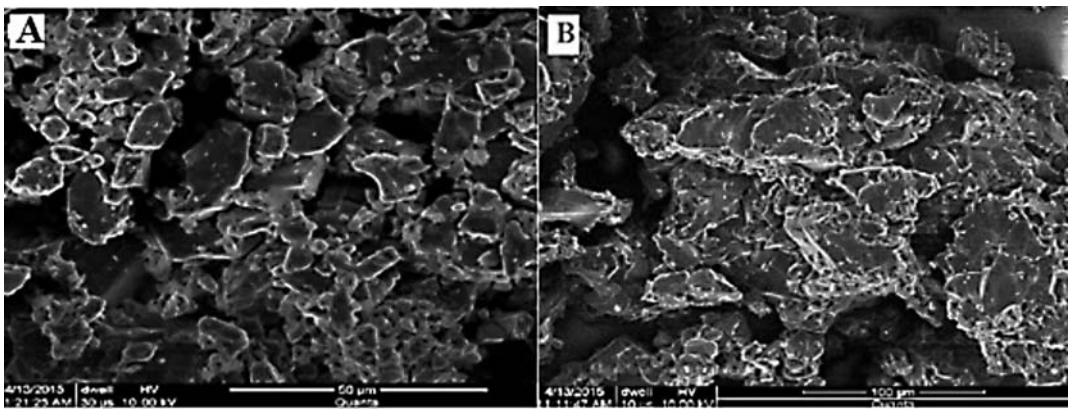


Figure 7. SEM images of acyclovir loaded inclusion complexes (**A**) and solid dispersions (**B**)

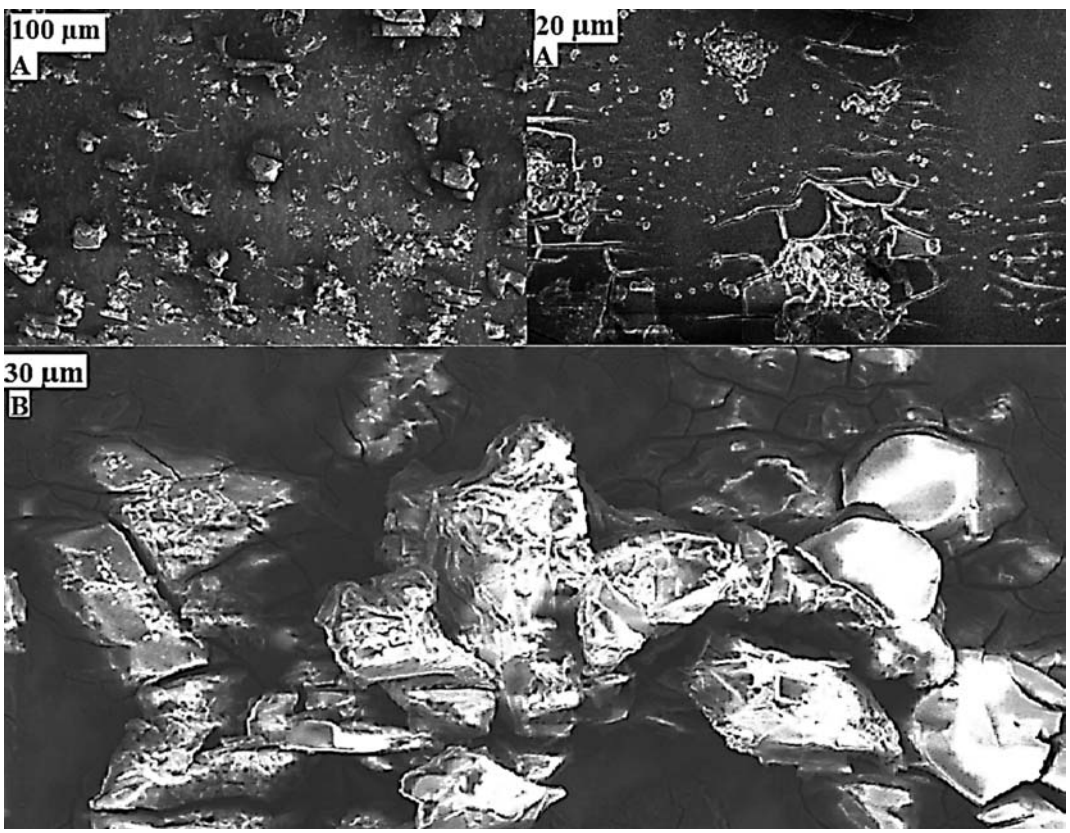


Figure 8. Transmission electron microscopy images of (**A**) inclusion complex IC3 and (**B**) solid dispersion SD3

was a decline in results of entrapment efficiency (32.18%) (11).

Micromertic properties

Angle of repose is associated with density, surface area and friction coefficient of tested powders. All prepared microparticles were having angle of repose 23.21 to 28.21^{o} that was less than official limit of 30^{o} exhibiting excellent flowability. Bulk and tapped densities proved better flow due to more dense particles, on the other hand, if particles are fine (< 100 µm) cohesiveness occurs. Hausner ratio was less than 1.25 confirming good flow of microparticles. Similarly, Carr's compressibility

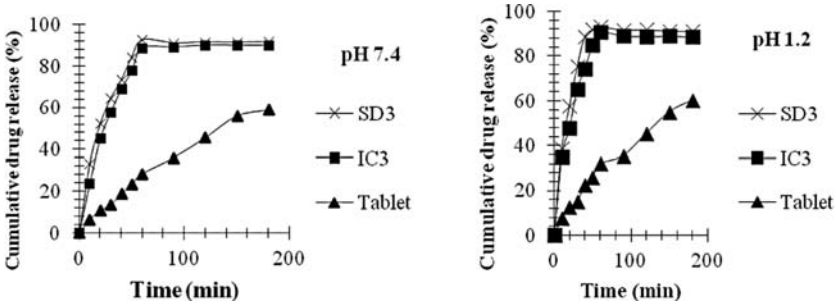
index values i.e., 16.03% to 19% also favored flow criteria. Results of micromeritic properties of Sarfraz et al. i.e., angle of repose (22.40 to 26.80°), bulk and tapped densities (less than 1 g/mL), Hausner ratio (less than 1.21) and Carr's index (14 to 17.51%) were according to official limits as in this study (12).

Particle size and zeta potential determination

Particle size was variable with polymer (β-CD) concentration, degree of agitation and stirring speed. Microparticles were of irregular shape but ranged within the micrometric scale (1-1000 µm). Microscopic evaluation also proved micro-sized particles. Due to smaller size and larger surface area, microparticles major part of atoms become closer to core and also become more reactive that ultimately pushes solubility profile to higher values. Zeta potential also effect particle size as it indicates repulsion between particles. Neutral values assure stability of products while lower values results in aggregation. It was neutral among 0 to 40000 counts thereby proving stability of microparticles. Stable microparticles can easily be dispersed that enhances the solubility of incorporated therapeutic moiety.

Fourier transform infrared spectroscopy

FTIR-spectrum of β-CD and ACV physical mixture, peaks position and shapes were carried out. In case of IR-spectrum of solid dispersions, C-OH absorptive peak was observed at 3287 cm^{-1}, C-H (2923 cm^{-1}), amine groups (1597 and 1697 cm^{-1}). IR spectrum of inclusion complexes have shown C-OH absorptive peak at 3306 cm^{-1} and amino groups have represented their identity at 1629 and 1717 cm^{-1}. These shifting of peaks demonstrated complex formation



Fiigure 9. *In vitro* dissolution studies at pH 1.2 and pH 7.4

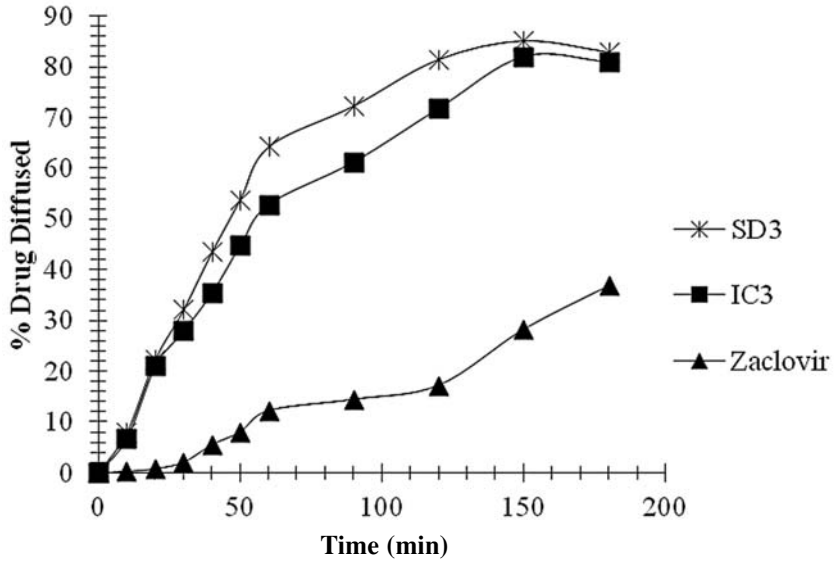


Figure 10. *Ex vivo* permeability studies

between ACV and β-CD. A study by Marinescu et al. revealed the presence of β-CD characteristic transmittance peaks at C-OH (3368 cm^{-1}) and C-H (2927 cm^{-1}). In complexed form peak shifting and variability in intensities was seen accordingly (16).

Thermal analysis

DSC of IC's revealed that exothermic peak at 81.25°C was absent and peak at 382.31°C was shifted to 380.04°C. In TGA there was only 0.44% mass loss in first 3.56 min, 4.22% up to 12.24 min and 15.46% in 13.88 min as compared to TGA of pure β-CD. In case of SD's, exothermic peak at 382.31°C was absent and peaks at 81.25°C, 324.95°C were shifted to 92.88°C and 242.22°C in DSC studies. In TGA of SD's, there was only 0.16, 7.20 and 14.14% mass loss occurred at 3.48, 10.77 and 14.19 min. ACV peak at 256.60°C was absent in both cases ensuring that it was encapsulated into β-CD cavity and stability was promoted.

Powder X-ray diffraction analysis (PXRD)

PXRD of formulated microparticles was performed to check status of crystallinity. Relatively, semi crystalline nature was seen due to peaks lower intensities in case of inclusion complexes, while in the case of solid dispersions, different patterns with least or uniform peak intensities were seen thereby proving amorphous state.

Scanning electron microscopy (SEM)

SEM images revealed that IC's and SD's were of irregular shape having enlarged surface area. Micrometric size range and high surface area occurred due to size reduction, which has prominent effect on solubility enhancement and rapid release of active ingredient.

Transmission electron microscopy (TEM)

Transmission electron microscopy proved successful loading of ACV in microparticles, size range within micrometric scale and shape of developed ACV microparticles.

Dissolution studies

Dissolution studies of all developed ratios were performed at pH 1.2 and pH 7.4 to check dependence upon solubility, degree of ionization, pKa values and also to evaluate release pattern in gastric fluid and in intestinal region. Higher release or % ACV in dissolved form was noticed in case of SD's i.e., 93.12 and 90.75% at pH 1.2 in the first hour. Slightly lower release results occurred in case of IC's i.e., 92.5 and 88.75% at pH 7.4, respectively, within first 60 min. At higher pH values, dissolution of ACV from SD's and IC's was increased as compared to free/pure ACV due to impact of β-CD and manufacturing techniques. So physical binding of ACV in the form of solid dispersions and inclusion complexes imparted a positive effect. *In-vitro* drug release kinetic declared first order model as best fit model depending upon R^2 value i.e., SD3 (0.9937), IC3 (0.9939) and Cap. Zaclovir (0.9928). According to Korsmeyer Peppas model, values of (n) were 0.254 (SD3) and 0.300 (IC3), while in the case of ACV tablet 0.764 so proving Fickian diffusion while tablets induces anamolus transport. Pedotti et al. concluded that dissolution profile of complexed ACV was greater at high pH when compared with pure ACV. Similarly, complexed ACV has shown even higher dissolution at pH 1.2 (19).

Permeability studies

SLS was incorporated in a fixed amount (4% of total weight of each formulation). β-CD enhances bioavailability of ACV at absorptive surface, while SLS causes opening of tight junctions, extract intracellular lipids or prevent active efflux ultimately promoting permeability of ACV across intestinal membrane. Moreover, at higher pH values ACV exists in unionized form that has more capability to cross intestinal mucosa. Higher permeability results were seen in case of SD's 85.18% while Zaclovir exhibited only 36.89% diffusion. Dias et al. have shown that by increasing surfactant concentration, permeability of ACV increases markedly due to an increase of partition coefficient that results in higher ACV content penetration (15).

CONCLUSION

Co-incorporation of β-CD and SLS into developed microparticles (SD's & IC's) significantly promoted solubility, bioavailability and permeability of acyclovir. Simple techniques i.e., kneading method and solvent evaporation technique were utilized that ultimately resulted in reduced cost of therapy, improvement of patient compliance and decrease of dosing frequency. This approach can also be utilized for other BCS Class IV drugs of particular interest.

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OPTIMIZATION OF FUROSEMIDE LIQUISOLID TABLETS PREPARATION PROCESS LEADING TO THEIR MASS AND SIZE REDUCTION

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Abstract: The great number of drug substances currently used in solid oral dosage forms is characterized by poor water solubility. Therefore, various methods of dissolution rate enhancement are an important topic of research interest in modern drug technology. The purpose of this study was to enhance the furosemide dissolution rate from liquisolid tablets while maintaining an acceptable size and mass. Two types of dibasic calcium phosphate (Fujicalin®/Emcompress®) and microcrystalline cellulose (Vivapur® 102/Vivapur® 12) were used as carriers and magnesium aluminometasilicate (Neusilin® US2) was used as a coating material. The flowable liquid retention potential for those excipients was tested by measuring the angle of slide. To evaluate the impact of used excipients on tablet properties fourteen tablet formulations were prepared. It was found that LS2 tablets containing spherically granulated dibasic calcium phosphate and magnesium aluminometasilicate exhibit the best dissolution profile and mechanical properties while tablets composed only with Neusilin® US2 was characterized by the smallest size and mass with preserved good mechanical properties and furosemide dissolution.

Keywords: liquisolid, dissolution rate, furosemide, solubility

Gastrointestinal absorption of a drug administered orally is affected by multiple parameters including drug dissolution rate, which is influenced by the drug solubility in gastrointestinal fluids. Water solubility of an active pharmaceutical ingredient (API) is the critical factor in the design of solid oral dosage forms. Nowadays, approximately 40% of the immediate release dosage forms administered orally contain active substances that are insoluble in water (1). Therefore, the low dissolution rate of poorly soluble drugs, i.e., belonging to the class II and IV of the Biopharmaceutical Classification System (BCS), still remains one of the major problems in the formulation of the dosage forms. Many strategies can be applied to improve drug dissolution properties, namely: micronization, formulation of solid dispersions, complexation with β-cyclodextrins or drug derivatization.

The liquisolid technique that has emerged in the last decade is a promising approach to the solubility improvement of poorly water soluble drugs. Its main advantages include simplicity of manufacturing, use of commercially available excipients, and application of well-known methods and equipment utilized for the manufacturing of conventional tablets.

Liquisolid systems (LCS) are described as dry, free flowing and compressible powder mixtures with absorbed solution or dispersion of drug substance in non-volatile solvent or liquid drug form (2). The liquisolid system is mainly composed of two groups of excipients: carriers and coating materials. Various grades of microcrystalline cellulose (MCC), dibasic calcium phosphate, pregelatinized starch and lactose are commonly used as carriers, while different types of silica dioxide are utilized as coating material (3). The factor limiting formulation of reasonable size and low mass liquisolid tablets is the solubility of the active substance in non-volatile solvents. Thus, the application of solvents characterized by high solubilizing capability could be a promising method of reducing tablet size and mass. The most commonly used solvents include propylene glycol, polyethylene glycol, polysorbate, Cremophor® EL or Synperonic™ PE/L61 (4).

Insoluble model drug substances such as carbamazepine, famotidine, furosemide, piroxicam and

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prednisolone have been used in the liquisolid technology development (3-7).

A crucial limitation factor in the application of LCS technology is the dose of drug substance. Javadzadeh et al. (6) suggested that the liquisolid method is suitable for dosages lower than 50 mg. Nevertheless, it was found that the application of polymeric additives, e.g., polyvinylpyrrolidone (PVP) might help to overcome this problem, and higher amounts of drugs can be used. In that study, 100 mg of carbamazepine was loaded into liquisolid tablets weighting from 583 mg to 1010 mg per unit, but there is a lack of information about the size of the tablets (6). The application of excipients with high absorption capacity is another possibility to decrease the mass of the tablets. It was shown that application of porous excipients such as magnesium aluminometasilicate instead of conventional tableting excipients resulted in an increased drug loading capacity of formulation (3). Despite the low dose of griseofulvin, 3 mg of the API had to be introduced in the form of 2 to 5 tablets with a diameter as big as 10 mm (8). The size and the mass of the tablets are the fundamental factors affecting patient compliance. In the case of liquisolid tablets, the attributes result from the dose of the API, its solubility in the solvent used, carrier and coating material absorptivity and their weight ratio as well as polymer additives to the formulation.

The aim of this study was to develop and optimize liquisolid tablets composition characterized by improved dissolution rate and possible small size and mass. Furthermore, the influence of various carriers and carrier to coating material ratio were studied in respect to the liquisolid tablets mechanical properties and model drug dissolution profile. Furosemide, in the dose of 40 mg which is registered on the market, was chosen as a model practically insoluble in water drug substance which belongs to biopharmaceutical classification system (BCS) class IV (9). So far, liquisolid formulations containing 20 mg of furosemide were studied by Akinlade et al. (7).

EXPERIMENTAL

Materials

Furosemide in the form of micronized powder (J.B. Chemicals and Pharmaceuticals, India) was used as a model drug. Microcrystalline cellulose of various grades (Vivapur® 102 and Vivapur® 12), anhydrous dibasic calcium phosphate - Emcompress® Anhydrous (JRS Pharma, Rosenberg, Germany) and spherically granulated anhydrous dibasic calcium phosphate Fujicalin® (Fuji Chemical Industries, Toyama, Japan) were used as carriers, magnesium aluminometasilicate Neusilin® US2 (Fuji Chemical Industries, Toyama, Japan) as a coating material, crospovidone - Kollidon® CL (BASF, Ludwigshafen, Germany) as a disintegrant and macrogol 400 (B.D.H. Chemicals) as a drug solvent.

Solubility studies

The solubility studies of furosemide were carried out in PEG 400 or in water. An excess amount of furosemide was added to 10 mL of solvent and shaken using a reciprocating shaker (IKA KS 130 BASIC, Germany) at 400 rpm for 24 h in room temperature to reach an equilibrium state. Then, the samples were centrifuged and filtered through a 0.45 μm Millipore filter, diluted and analyzed spectrophotometrically (Jasco V-530 UV/Vis spectrophotometer, Japan) at λ = 228 nm. All measurements were done in triplicate.

Excipients properties

The angle of repose was determined in accordance to Ph. Eur. 8.0 method by pouring the powder sample from the bottom sieve container mounted 70 mm above a round horizontal surface (60 mm dia.) until the cone was formed. The angle of repose was measured using measuring rod connected with the angular and metric scale. The angle of repose corresponds to the maximum angle between the slope of the formed cone and the horizontal surface. The attribute describes frictional forces in loose powder. Bulk and tapped density were measured according to the European Pharmacopeia using a W-1 volumeter (ZDM Polfa, Poland). Hausner ratio (HR) and Carr's index (CI) were calculated as follows:

$$CI = \frac{Tapped\ density - Bulk\ density}{Tapped\ density} \quad \text{(Eq.1)}$$

$$HR = \frac{Bulk\ density}{Tapped\ density} \quad \text{(Eq. 2)}$$

The moisture content was analyzed using a RadWag WPE 30S apparatus at 100°C and 15 s sampling time till the constant weight of the sample was achieved.

The flowable liquid retention potential (Φ) (Eq. 3) corresponds to the maximum amount of liquid that can be absorbed by the excipient, while preserving its good flowability (10). To obtain the liquisolid powder with good flow properties, the angle of slide of the excipients with admixture of increasing amounts of PEG 400 was investigated. The sample of powder (1.0 g) was placed on a polished metal plate which was gradually tilted until the

sample started to slide. In order to check physical properties of admixed ingredients the samples were examined 5 min and 24 h after preparation. Values of Φ were calculated for mixtures which slides down at the angle of plate inclination of 33°, which is considered to be optimal flow of the powder.

$$\Phi = \frac{W_{liquid}}{W_{powder}} \quad \text{(Eq. 3)}$$

where: W_{liquid} – weight of liquid, W_{powder} – weight of dry powder.

Preparation of liquisolid tablets

The composition of tablets is presented in Table 1. The maximum amount of furosemide solution (liquid load factor L_f) which can be loaded into powder bulk was calculated according to equation 4 (10):

$$L_f = \Phi_{carr.} + \Phi_{coat}\ (1/R) \quad \text{(Eq. 4)}$$

where: – liquid load factor, $\Phi_{carr.}$, $\Phi_{coat.}$ – flowable liquid retention potential for carrier and coating material, respectively, R – carrier/coating material ratio.

Twelve liquisolid formulations with four type of carriers: Fujicalin® (LS1 – LS3), Emcompress® (LS4 – LS6), Vivapur® 12 (LS7 – LS9) and Vivapur® 102 (LS10 – LS12) were prepared. The each individual carrier was combined with different amount of coating material. In particular, the following carrier to coating material ratios were examined: 1 : 1, 5 : 1, 10 : 1. Tablets were prepared as follows, the carrier was placed in a mortar and mixed with 20% furosemide solution in macrogol 400 poured in the quantity equal to 40 mg dose of the API. The coating substance (Neusilin® US2) was gradually added and blended gently for 5 min. Then, 5% of Kollidon® CL was added and mixing was continued for the next 5 min. Final blends were compressed using a Korsch EK0 single punch tablet press (Germany). Two lots of tablets (LS13 and LS14) were prepared using magnesium aluminometasilicate in the function of carrier and coating material. The lots were differentiated by quantities of disintegrant as follows: 5% for the LS13 formulation and 10% for LS14.

Preparation of directly compressed tablets (DCT)

Control tablets containing 40 mg of crystalline furosemide were prepared with direct compression method. The furosemide powder was mixed with suitable amounts of considered carrier and coating material. Afterwards, 5% of Kollidon Cl was added as a disintegrant and mixed. Final blend was compressed using Korsch EK0 (Germany) eccentric tablet press.

Evaluation of tablet properties

Tablet mass uniformity, thickness and hardness

Ten tablets of each formulation were accurately weighed and measured. The hardness of the tablets was evaluated using a VanKel VK 200 hardness tester (USA).

Table 1. Composition of prepared liquisolid tablets calculated on 100 tablets batch.

Form.	Carrier (Q) quantity [g]		Coating material quantity [g]	20% furosemide solution [g]	Kollidon Cl amount [%]	R	L_f
LS1	Fujicalin	11.2	11.2	20.0	5%	1	1.793
LS2		33.2	06.6			5	0.602
LS3		44.2	4.4			10	0.453
LS4	Emcompress	11.9	11.9			1	1.678
LS5		41.1	08.2			5	0.487
LS6		59.1	5.9			10	0.338
LS7	Vivapur 12	12.7	12.7			1	1.574
LS8		52.2	10.4			5	0.383
LS9		85.4	8.5			10	0.234
LS10	Vivapur 102	13.2	13.2			1	1.510
LS11		62.7	12.5			5	0.319
LS12		117.5	11.8			10	0.170
LS13	Neusilin US2	13.4			5%	-	-
LS14		13.4			10%	-	-

Friability

The friability test was performed according to the European Pharmacopoeia 8.0 using PharmaTest PTF-E friabilator (Germany).

Disintegration time

Tablets disintegration time was determined using a disintegration test ElectroLab ED2 Sapo (India) apparatus in accordance with Ph. Eur. 8.0 method. Purified water kept at 37°C was used as a medium, six randomly selected tablets from each formulation were evaluated.

Furosemide content determination

Three randomly taken tablets of each formulation were accurately weighed and shaken with 200 mL of sodium hydroxide solution (4 g/L) over 24 h (IKA KS 130 Basic shaker, Germany). Afterwards, samples were centrifuged at 3600 rpm and filtered through 0.45 μm Milipore® filter. After dilution, drug concentration was assayed spectrophotometrically using a Jasco V-530 UV-Vis spectrophotometer (Japan) with a wavelength λ = 228 nm.

Dissolution studies

Drug dissolution studies were carried out using a Ph. Eur. 8.0 dissolution apparatus 2 (Hanson Research SR8 Plus Dissolution Test Station, USA) operated at 50 rpm. Tablets were placed in 900 mL of 0.1 mol/L hydrochloric acid solution (pH 1.2) at 37°C. Sample volumes of 5 mL were withdrawn from each dissolution vessel at 5, 10, 15, 30, 60 and 120 min and analyzed spectrophotometrically at λ = 228 nm. Every time vessel volumes were replen-

Table 2. Excipients parameters.

Excipient	Φ value	Humidity content (%)	Angle of repose (°)	Hausner ratio	Carr index (%)
Vivapur 102	0.021	4.7	45	1.38	27.7
Vivapur 12	0.085	2.5	38	1.39	28.3
Emcompress Anh.	0.189	0.6	34	1.21	17.0
Fujicalin	0.304	1.1	30	1.14	12.5
Neusilin US2	1.489	7.2	27	1.15	13.3

Table 3. Physical parameters, disintegration time and amount of drug dissolved of each formulation.

Form.	Tablet mass [mg] ± SD	Dia. [mm]	Thickness [mm]	Hardness [N] ± SD	Friability [%]	Average disintegration time	Amount of API dissolved after 2 h [%] ± SD
LS1	439.1 ± 8.6	10	4.07	45.6 ± 7.3	0.78	5 min 11 s	83.11 ± 5.77
LS2	627.7 ± 11.9	11	4.92	38.5 ± 2.4	0.13	1 min 58 s	92.93 ± 10.54
LS3	721.2 ± 6.3	12	4.25	48.4 ± 2.5	0.03	7 min 40 s	91.08 ± 7.29
LS4	463.7 ± 9.5	11	3.60	40.3 ± 5.5	0.31	9 min 07 s	85.17 ± 18.84
LS5	728.7 ± 8.1	11	4.68	51.3 ± 3.2	0.06	5 min 22 s	83.59 ± 14.06
LS6	833.8 ± 14.5	11	5.43	56.7 ± 9.1	0.08	1 min 26 s	84.79 ± 9.59
LS7	476.4 ± 8.7	15	3.74	40.6 ± 0.9	0.15	9 min 18 s	79.11 ± 15.53
LS8	870.0 ± 14.9	15	4.45	53.5 ± 9.3	0.15	29 s	69.27 ± 1.16
LS9	1200.0 ± 13.3	15	6.88	58.3 ± 4.5	0.97	26 s	71.09 ± 2.16
LS10	484.9 ± 5.8	12	3.68	48.2 ± 3.6	0.17	12 min 13 s	52.18 ± 17.66
LS11	1009.0 ± 11.2	15	5.75	53.6 ± 3.3	0.79	31 s	76.21 ± 0.49
LS12	1569.0 ± 34.1	20	4.93	48.5 ± 7.4	0.71	24 s	68.20 ±3.30
LS13	349.7 ± 5.6	10	3.88	36.2 ± 4.0	0.13	14 min 53 s	85.83 ± 6.55
LS14	360.0 ± 6.8	10	4.16	37.2 ± 5.7	0.14	7 min 51 s	95.86 ± 5.66
DCT	460.0 ± 7.1	12	4.21	58.35± 4.2	0.90	52 s	28.53 ± 2.30

ished automatically with fresh medium. The measurements were carried out in triplicate. There was no interference on the absorption spectrum of furosemide from liquid vehicles and other excipients.

RESULTS AND DISCUSSION

The solubility of furosemide in macrogol 400 (234.97 mg/mL) was approximately 10000 times higher than in water (26.9 μg/mL). Javadzadeh et al. (12) showed that the dissolution rate is directly proportional to the fraction of molecularly dispersed drug (F_M) and the use of drug solution causes that the F_M factor is as high as possible (F_M = 1). The high solubility of furosemide in macrogol 400 allows obtaining small tablets since less amount of liquid has to be adsorbed onto powder surface.

According to the flowability results obtained for raw materials it was stated that Neusilin® US2 possesses the best flow properties (Table 2). Its flow characteristic can be expressed as excellent based on the angle of repose lower than 30° and good according to the compressibility index (11-15%) and Hausner ratio (1.12 – 1.18). The results of the liquid sorption capacity show that the values of flowable liquid retention potential (Phi, Φ) were correlated with the angle of repose of raw material and it increases with lower angle of repose values. The Phi-values were in the range from 0.021 for Vivapur® 102 up to 1.489 for magnesium aluminometasilicate, which corresponds with the highest angle of repose of Vivapur® 102 (45°, passable flow - may hang up) and the lowest for Neusilin® US2 (27°, excellent flow) from all of tested excipients. The high humidity content of Neusilin® US2 is due to its large specific surface area reaching up to 300 m^2/g (11), but absorbing capacity was not affected by the humidity content, because Φ-value was the highest and reached up 1.49. The comparison of Emcompress® and Fujicalin® shows better flow properties of the spherically granulated dibasic calcium phosphate (Fujicalin®). The difference between those excipients is in their specific surface area and shape. Fujicalin® is composed of spherically granulated particles with a porous surface which results in a high specific surface area, i.e., over 32 m^2/g while Emcompress® Anhydrous has 20.7 m^2/g (11) which resulted in approx 1.6-fold lower liquid sorption capacity. Also the microcrystalline cellulose Vivapur® 12 with a larger particles, of median size 180 μm, has a higher value of flowable liquid retention potential in comparison to Vivapur® 102 with an average particle size of 100 μm. Based on the comparison of obtained results it can be stated that the spherically granulated anhydrous dibasic calcium phosphate is the most suitable carrier.

The characteristics of all kinds of tablets are presented in Table 3. Among the evaluated physical properties such as mass, thickness, hardness, friability and disintegration time for liquisolid formulations, the differences has been identified particularly in tablet mass and disintegration time. A relationship between the excipients properties and the tablets mass and diameter was identified. Tablets prepared with high absorption capacity substances such as magnesium aluminometasilicate (LS13, LS14) and spherically granulated anhydrous dibasic calcium phosphate i.e. formulations LS1–LS3 have the lowest mass among of the studied formulations prepared with different types of carriers. When Emcompress® was used as a carrier the increase of tablet mass was estimated while disintegration time was elongated only for two formulations (LS4, LS5). Tablets with microcrystalline cellulose PH102 were characterized by the highest mass exceeding 1569 mg and use of punches with 20 mm in diameter (LS12). Similar results were obtained by Hentzschel et al. while replacing microcrystalline cellulose and silica with Neusilin® US2 causing the griseofulvin unit dose mass reduction from 2026 to 600 mg (8).

The tablets parameters also depend on carrier/coating material ratio. The increase of Neusilin® US2 amount in the formulation results in the tablet mass and size decrease due to its high sorption capacity. For example, the mass of LS1 tablets containing 112 mg of Neusilin® US2, with the carrier to coating ratio 1 : 1, was 439 mg and 10 mm in diameter while in the case of LS3 tablets, in which the amount of Neusilin® US2 was three times lower, the tablet mass was 721 mg and diameter was 12 mm. The same relationship was identified for tablet formulations prepared with other carriers. Based on Ph. Eur. 8.0 monograph all of the tablets met the pharmacopeial friability requirement because the loss of weight after the friability test did not exceed 1%. The average disintegration time ranged from 24 s (LS12) to 14 min 53 s (LS13). Compared the average disintegration times for tablets containing microcrystalline cellulose with different amount of Neusilin® US2 i.e., LS7 – LS12 tablets, it was stated that high quantity of Neusilin® US2 in formulation significantly affect the disintegration time. This was confirmed by long disintegration times for tablets composed only with this coating material. LS13 tablets do not comply with the pharmacopeia

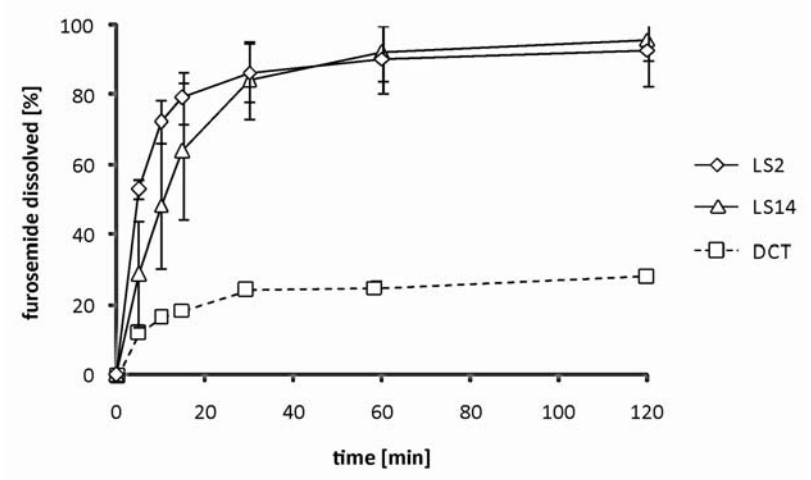

Figure 1. Comparison of furosemide dissolution profiles for the best liquisolid tablets and direct compressed control tablets

requirement, despite that the average time was below 15 min but three of the six tablets had a disintegration time over 15 min. Two-fold higher amount of the disintegrant in the LS14 formulation with respect to the LS13 tablets resulted in reduction of disintegration time by half (Table 3).

The effect of the excipients used in tablet formulations on furosemide release profile was investigated. As shown in Table 3, there were large differences in drug dissolution between fourteen types of tablets. The amount of furosemide dissolved after 2 h was in the range from 52.18% to 95.86%. Taking into account tablet formulations prepared with both kinds of the excipients i.e., carrier and coating material (LS1 – LS12), the highest amount of the drug (92.93%) was released from LS2 liquisolid tablets containing spherically granulated anhydrous dibasic calcium phosphate (Fujicalin®) as a carrier. In the case of Emcompress®, independently of excipients quantity the amounts of furosemide released after 2 h were similar 83.6% – 85.2% (formulations LS4 – LS6). The release profiles of furosemide for microcrystalline cellulose based formulations varied depending on the physical properties of the carrier, and Vivapur® to Neusilin® US2 ratio (Table 3).

The dissolution profiles of furosemide from tablets with 1 : 1 carrier/coating ratio (LS1, LS4, LS7) were similar independently from the kind of the carrier i.e., Fujicalin®, Emcompress® and Vivapur® 12. The only exception was the formulation with Vivapur® PH102 (LS10). This kind of tablets differs in the physical properties and dissolution of furosemide. They were characterized by long disintegration time, exceeding 12 min and the lowest amount of furosemide dissolved after 2 h (52.2%). High quantity of Neusilin® US2 with its large specific surface area significantly influenced furosemide dissolution profile.

From these results it can be assumed that liquisolid tablets with Fujicalin® and Neusilin® US2 are recommended to enhance the dissolution rate of furosemide. Two formulations LS2 and LS14 fully met the formulation expectations. The similar amount of furosemide was released from both LS2 and LS14 formulations after 2 h i.e., 92.93 and 95.86%, respectively. The mass of LS2 tablets was 627 mg, disintegration time 1 min 58 s. In comparison, the mass of the LS14 tablets was the smallest one i.e., 366 mg but the disintegration time was 4-fold longer. As it is presented on Figure 1, the amount of dissolved drug after 2 h from liquisolid tablets was over 3-times greater than in control tablets (DCT).

CONCLUSION

This study showed that liquisolid technique is a promising strategy in the pharmaceutical technology. The formulation of liquisolid tablets enhances the dissolution rate of furosemide when compared with direct compressed control tablets. Typically used dose of furosemide is 40–120 mg a day but there was no study on 40 mg furosemide liquisolid tablets. The results of investigations demonstrate that by selection of suitable excipients 40 mg furosemide tablets with acceptable size and mass with fast and entire drug dissolution could be prepared. Spherically granulated dibasic calcium phosphate (Fujicalin®) and magnesium aluminometasilicate (Neusilin® US2) as carrier and coating materi-

al, respectively, are suitable for liquisolid technique. The optimized formulations LS2 and LS14 showed 86.5 ± 8.6% and 84.3 ± 11.4% drug release within first 30 min and 92.9 ± 10.5% and 95.9 ± 5.7% after 2 h, respectively. The improvement in the furosemide dissolution characteristics from liquisolid tablets is mainly due to utilization of solution of the API. It was also stated that the dissolution profiles are affected by the amount of highly absorptive excipients such as Neusilin® US2. Other physical parameters such as hardness, friability and disintegration time were also satisfactory. The mass of both formulations was also reduced. It is an important factor that should be considered in aspect of patient compliance.

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LEVEL A *IN VITRO-IN VIVO* CORRELATION DEVELOPMENT AND VALIDATION FOR TRAMADOL HYDROCHLORIDE FORMULATIONS

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Abstract: The objective of this article is to develop and validate the level A *in vitro-in vivo* correlation (IVIVC) for three different formulations of tramadol hydrochloride. The formulations included were Tramazac® (M1, conventional tablet) and TRD CONTIN® (M2, sustained release tablet), and a new controlled release tablet prepared on the basis of osmotic technology (formulation IVB). To develop level A IVIVC, *in vivo* data were deconvoluted into absorption data by using Wagner-Nelson equation. The absorption data (percent drug absorbed) was plotted against percent drug dissolved keeping the former along x-axis and the later along y-axis. The highest determination coefficient (R^2 = 0.9278) of the level A IVIVC was observed for formulation M1, and then for M2 (R^2 = 0.9046) and IVB (R^2 = 0.8796). Additionally, plasma drug levels were approximated from *in vitro* dissolution data using convolution approach to calculate the prediction error (%), which was found to be < 10%.

Keywords: tramadol hydrochloride, controlled release, osmotic technology, deconvolution, convolution, *in vitro-in vivo* correlation

The dissolution is the rate limiting step for BCS class I drugs, thus level A *in vitro-in vivo* correlation (IVIVC) for modified release formulations of these drugs is expected in view of Food and Drug Administration (FDA) guidelines (1). Being point-to-point correlation, level A IVIVC is considered the strongest correlation, which is useful to predict the *in vivo* absorption fate of drug from its *in vitro* dissolution data. It has urged the need for developing IVIVC for various drugs (2).

Tramadol hydrochloride, in this regard, is a potential candidate for preparing the modified release formulation and developing IVIVC. Tramadol hydrochloride is indicated for management of severe pains. This drug has good oral bioavailability. Moreover, its elimination half-life is 5–7 h and it administered thrice a day (3). The patient non-compliance due to frequent dosing schedule, particularly in long-term use, can be avoided through development of the controlled release system for tramadol hydrochloride (4).

Level A IVIVC for tramadol hydrochloride has already been developed in various studies (5), but there is no such study that involve its osmotic formulation(s). This article is an extension of previous publication regarding fabrication of prolonged release formulation of tramadol hydrochloride based on osmotic technology. The osmotic formulations have many applications in drug delivery owing to pH-independent drug release (6, 7).

In order to develop level A IVIVC, FDA suggests to use two or more than two formulations of a drug having different release rates. Moreover, the release rates as determined by percent drug dissolved, and *in vivo* (pharmacokinetic) parameters (C_{max} or AUC) for each formulation studied, should be different sufficiently, for example by 10% (5).

This piece of writing is a component of previous study (8), conducted for the formulation of controlled delivery system for tramadol hydrochloride based on osmotic technology. Out of the designed formulations, the optimum one was further tested to get *in vivo* data for the development of *in vitro-in vivo* correlation along with the reference formulations, i.e., Tramazac® (M1, conventional tablet, Zydus Cadila Pharmaceuticals, USA) and TRD

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CONTIN® (M2, sustained release tablet, Modi-Mundi Pharmaceuticals, USA).

EXPERIMENTAL

Materials

Tramadol hydrochloride (Win Medicare, USA) and polyethylene oxide (Torrent Research Centre, USA) were obtained as gift samples. Methanol, acetone, acetonitrile, triethanolamine, fructose, and mannitol were procured from Qualigens Fine Chemicals, USA. Polyvinyl pyrrolidone (PVP K-30), cellulose acetate (39.8% acetylation), microcrystalline cellulose (MCC PH 102), magnesium stearate, talc, and sodium chloride were purchased from CDH, USA. Disodium hydrogen orthophosphate and orthophosphoric acid were obtained from S.D. Fine Chemicals, USA. Marketed tablets of tramadol hydrochloride, i.e., Tramazac® (M1, conventional tablet, Zydus Cadila Pharmaceuticals, USA) and TRD CONTIN® (M2, sustained release tablet, Modi-Mundi Pharmaceuticals, USA) were purchased from a retail pharmacy. All chemicals and excipients were analytical in nature, purchased from commercial sources and used without any modification.

Formulation development

Formulation development involved the preparation of core tablets of tramadol hydrochloride by direct compression technique (Table 1). The amount of tramadol hydrochloride (100 mg/tablet), PVP K-30 (10 mg/tablet), talc (2 mg/tablet), and magnesium stearate (2 mg/tablet) were kept constant. All the batches contained an average mass of 300 mg, except batch V, which contained 320 mg mass. After sieving, all the materials used in core formulation development were mixed with each other in the said amounts. This mixture was then converted into tablets by applying suitable compression force using tablet punching machine (Manesty E-2, UK). Then, coating was applied to these prepared core tablets using conventional laboratory coating pan (Scientific Instrument, USA). The coating solutions, coded as A, B, and C, contained PEG 400 (mg) in an amount of 0, 400, and 800 mg, respectively. In addition, all coating solutions contained 4000 mg, 10 mL, and 90 mL of cellulose acetate, methanol, and acetone, respectively. After applying coating of desired thickness (150 mm), an aperture of 0.5 mm was made on one face of all the coated tablets using a microdrill (Kamlesh Engineers, USA), followed by oven-drying of the formulations (8).

In vitro characterization

In vitro characterization of the powdered blend involved bulk and tap density, compressibility index and Hausner's ratio determination (9). Moreover, *in vitro* characterization of the prepared tablets included the assessment of their weight variation, hardness, thickness, diameter, and content uniformity (10). Additionally, *in vitro* dissolution testing of the formulated tablets was conducted in simulated intestinal fluid (pH 6.8, 900 mL) maintained at 37 ± 0.5°C using a USP dissolution apparatus type II (Campbell Electronics, USA) stirred at 50 rpm. At pre-determined time intervals, an adequate volume of 5 mL was withdrawn as the dissolution samples replacing with same volume of fresh medium. The dissolution samples were tested at 271 nm using a reported validated HPLC modality (8) to determine the concentration of drug released, and then the drug release (%) was plotted against time. These dissolution profiles of different formulations were compared with each other using similarity factor, f_2. It reveals that the two dissolution profiles are deemed to be similar if f_2-value ranges between 50 and 100 (11).

In vivo characterization and *in vitro-in vivo* correlation development

A HPLC system (Cecil, UK) consisting of dual piston pump (CE-4100, UK), manual injector (Caplugs RC-11, Germany), UV/Vis detector (CE-4201, UK), reverse phase C-18 column (4.6 × 250 mm, 5 μm) (Phenomenex, Germany) at 25°C was used.

Table 1. Concentrations of various formulation variables in different batches of core formulation.

Ingredients (mg per tablet)	Formulation codes				
	I	II	III	IV	V
Mannitol	-	136	136	136	136
Fructose	136	-	-	-	-
Polyethylene oxide	-	-	30	50	70
Microcrystalline cellulose	50	50	20	-	-

The optimized chromatographic conditions involved the solvent mixture of phosphate buffer (0.01 mol/L, 75%)/acetonitrile (25%)/tri-ethanolamine (0.1%) as mobile phase with a flow rate of 1 mL/min, 20 µL injection volume, and 271 nm wavelength for detection of drug (8).

Six healthy human subjects (weight range 55–75 kg, age range 24-29) of either sex participated in this crossover study with a wash-out period of 7 days. Signed consent letter was obtained from all the subjects that they were aware of the experimental protocol, which was approved by the human ethical committee, Banaras University. Pre- and post-dose heparinized blood samples were collected at the pre-determined time points, i.e., 0, 0.5, 1, 2, 4, 6, 8, 10, 12 and 24 h, followed by their storage at -4°C. At the time of analysis, liquid-liquid [NaOH (1 mol/L) – ethyl acetate] extraction based procedure (8) was adopted to extract proteins from plasma samples.

Finally, level A *in vitro-in vivo* correlation (IVIVC) for formulation IVB, M1, and M2 was developed. The development of level A IVIVC involves the use of Wagner-Nelson equation (12) to determine percent drug absorbed (F_a) and then plot a curve between F_a and F_d (percent drug dissolved) keeping the former along x-axis and the later along y-axis.

$$F_a = \{[C_t + (K_e \times AUC_{0\text{-}t})] / [K_e \times AUC_{0\text{-}\infty}]\} \times 100 \quad (1)$$

where, C_t is the drug plasma concentration at time *t*, K_e is elimination rate constant, $AUC_{0\text{-}t}$ is zero moment areas under the curve, and $AUC_{0\text{-}\infty}$ is first moment area under the curve (12).

Table 2. Determination coefficient (R2) and Y-equation for formulation M1, M2, and IVB

Formulation	Determination coefficient (R^2)	Y-equation
M1	0.9278	0.9803X + 14.125
M2	0.9046	0.7597X + 16.508
IVB	0.8796	0.7724X - 4.0379

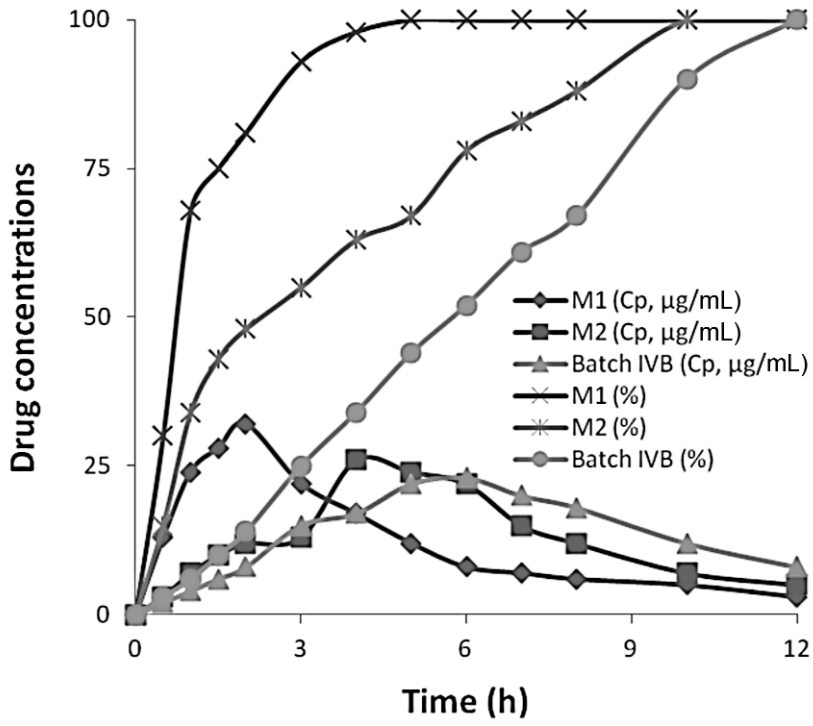

Figure 1. *In vitro* drug release pattern as well as the *in vivo* drug absorption data for formulation M1, M2, and IVB

For determining F_a, area under the plasma concentration-time curve from zero to time "t" (AUC_{0-t}), area under the plasma concentration-time curve from zero to time infinity ($AUC_{0-\infty}$), and elimination rate constant (K_e) were calculated leading to determination of two products, i.e. $K_e \times AUC_{0-t}$ at each time-point and $K_e \times AUC_{0-\infty}$. The former product was added to the respective C_t for the approximation of $C_t + (K_e \times AUC_{0-t})$, which was divided by $K_e \times AUC_{0-\infty}$. The result was multiplied by 100 at each time point to get F_a (%)(12).

To assess the strength of correlation (or to determine whether the level A IVIVC curve is linear or non-linear), level A IVIVC curves were evaluated by conducting their regression analysis, i.e., the correlation is considered stronger as well as the curve is considered linear, when the value of determination coefficient is closer to one (13).

The predictability of level A IVIVC was checked by using the following formula:

$$Prediction\ error\ (\%)_{C_{max}} = \frac{C_{max.observed} - C_{max.predicted}}{C_{max.observed}} \quad (2)$$

where, $C_{max.Predicted}$ was determined by convoluting the *in vitro* dissolution data for the establishment of plasma drug levels. This calculation involves the estimation of c(t) (predicted plasma drug concentration) from the *in vitro* dissolution data, (unit impulse response), the function *"u"* (the variable of integration), and (drug input rate *in vitro* from oral solid dosage form) (14). All these parameters jointly constitute the following equation of convolution:

$$C(t) = \int_0^t C_\delta(t-u)\, X'_{vitro}(u)du \quad (3)$$

The convolution approach involves various steps for the prediction of plasma drug concentration from the *in vitro* dissolution profiles. In first step, the percentage *in vitro* dissolution values during each sampling interval are converted into the distinct drug concentrations. These quantities are alternatively changed into the bioavailable drug concentrations and then, to the reducing levels of plasma drug concentrations using the literature on bioavailability of the studied drug. In this way, the predicted drug concentration values at each time point are obtained. These discrete drug levels are then added to get the predicted plasma drug profile at each time point (14).

RESULTS AND DISCUSSION

In vitro characterization

The effect of formulation variables on dissolution behavior of all developed formulations of tra-

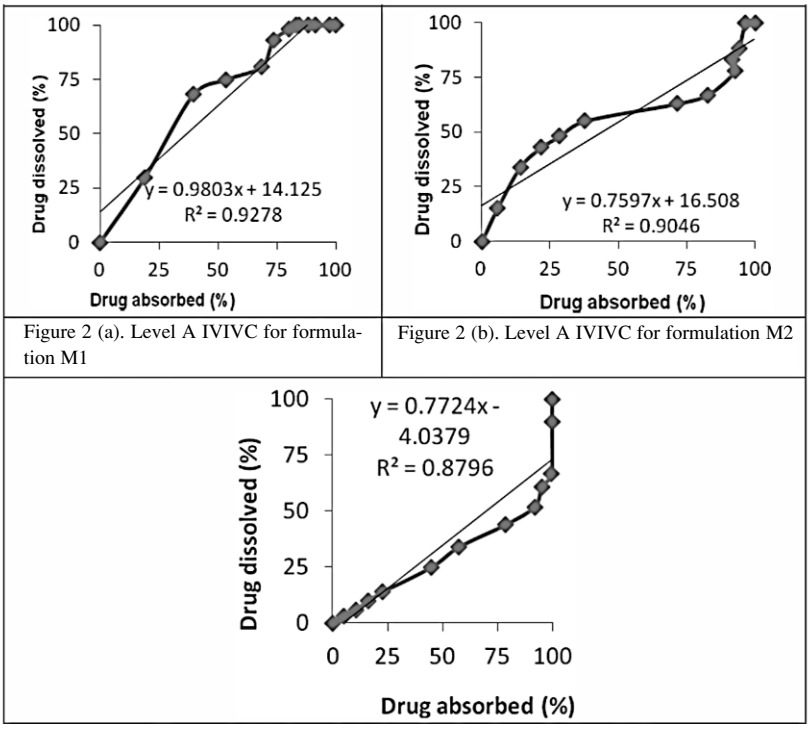

Figure 2 (a). Level A IVIVC for formulation M1

Figure 2 (b). Level A IVIVC for formulation M2

Figure 2 (c). Level A IVIVC for formulation IVB

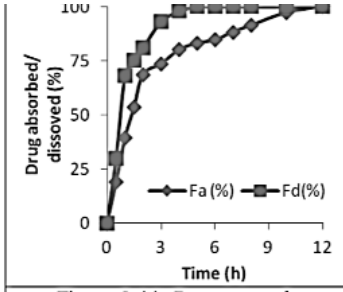

Figure 3 (a). Percentage drug dissolved and absorbed profiles *versus* time for formulation M1

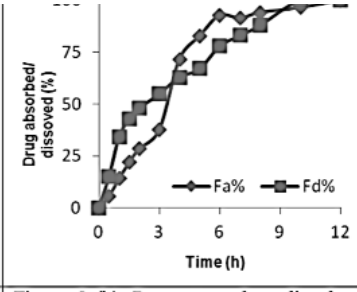

Figure 3 (b). Percentage drug dissolved and absorbed profiles *versus* time for formulation M2

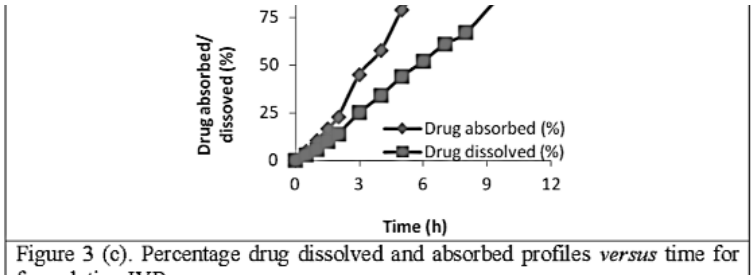

Figure 3 (c). Percentage drug dissolved and absorbed profiles *versus* time for formulation IVB

madol hydrochloride has already been documented (8). It was noticeably apparent from the dissolution curves that drug release from all developed formulations occurred in a controlled manner. Moreover, on comparing the *in vitro* dissolution profiles, drug release phenomenon from the developed formulations was found more controlled in comparison to that of commercial formulations M1 and M2. In addition, drug release from formulation IVB occurred in a desired controlled pattern as evident from fitting of zero-order equation; therefore, formulation IVB was picked as the optimum formulation.

From further analysis of this optimum formulation, it was found that the values of compressibility index and Hausner's ratio for the free flowing powder mixture of formulation IVB were < 15 and < 1.25, respectively. Additionally, the *in vitro* dissolution profile of this optimum formulation obtained by using simulated intestinal fluid was compared with that of achieved by adopting the pH change method. The results showed f_2 value of 91.85 that revealed the identical nature of both dissolution profiles.

In vivo characterization and *in vitro-in vivo* correlation development

The optimum formulation was further characterized for *in vivo* data to develop IVIVC along with M1 and M2. The *in vivo* data showed that the therapeutic tramadol hydrochloride plasma level from formulation IVB prolonged for more than 24 h, while formulations M1 and M2 could not maintain therapeutic tramadol hydrochloride plasma level even up to 24 h. The C_{max} value of formulation IVB was lower than that of formulations M1 and M2, which revealed the safe nature of formulation IVB in plasma. Moreover, t_{max} value of formulation IVB was higher than that of formulations M1 and M2, showing the controlled drug release behavior of formulation IVB, which also had higher values of $AUC_{0\text{-}24}$, relative bioavailability and mean residence time.

This article illustrates the assessment and mode of application of a biowaiver approach, IVIVC, in defining the *in vitro* release pattern of a formulation. The biowaiver studies depend on the *in vitro* dissolution data and can be conducted by using formulations with different strengths or release rates (4). Once an IVIVC is developed, the *in vivo* activity of a formulation can be predicted from its dissolution data. Moreover, IVIVC is also useful in bioequivalence studies and scale-up or post approval changes.

Figure 1 expresses the *in vitro* release pattern as well as the *in vivo* absorption data for the tested formulations. Level A IVIVC for formulation M1, M2, and IVB has been presented in Figure 2. Figure 3 shows the percentage drug dissolved and absorbed profiles *versus* time for formulation M1, M2, and

IVB. As a result of determining an IVIVC of formulation M1, M2, and IVB by applying Wagner-Nelson approach, a good linear regression was found from curves plotted between percent drug dissolved (along x-axis) and percent drug absorbed (along y-axis) (Table 2). The highest determination coefficient ($R^2 = 0.9278$) of the IVIVC was observed for formulation M1, and then for M2 ($R^2 = 0.9046$) and IVB ($R^2 = 0.8796$). Moreover, prediction error (%) for this biowaiver study was found to be < 10%.

CONCLUSIONS

It is clear from the results that strong *in vitro-in vivo* correlation exists between the *in vitro* release pattern as well as the *in vivo* absorption data for the tested formulations of tramadol hydrochloride, mainly for M1. Thus, level A *in vitro-in vivo* correlation is a good mathematical approach for its biowaiver evaluations.

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PHYSICAL AND CHEMICAL STABILITY ANALYSIS OF COSMETIC MULTIPLE EMULSIONS LOADED WITH ASCORBYL PALMITATE AND SODIUM ASCORBYL PHOSPHATE SALTS

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Abstract: Stability of hydrophilic and lipophilic vitamin C derivatives for quenching synergistic antioxidant activities and to treat oxidative related diseases is a major issue. This study was aimed to encapsulate hydrophilic and lipophilic vitamin C derivatives (ascorbyl palmitate and sodium ascorbyl phosphate) as functional ingredients in a newly formulated multiple emulsion of the W/O/W type to attain the synergistic antioxidant effects and the resultant system's long term physical and chemical stability. Several multiple emulsions using the same concentration of emulsifiers but different concentrations of ascorbyl palmitate and sodium ascorbyl phosphate were developed. Three finally selected multiple emulsions (ME_1, ME_2 and ME_3) were evaluated for physical stability in terms of rheology, microscopy, conductivity, pH, and organoleptic characteristics under different storage conditions for 3 months. Chemical stability was determined by HPLC on Sykam GmbH HPLC system (Germany), equipped with a variable UV detector. Results showed that at accelerated storage conditions all the three multiple emulsions had shear thinning behavior of varying shear stress with no influence of location of functional ingredients in a carrier system. Conductivity values increased and pH values remained within the skin pH range for 3 months. Microscopic analysis showed an increase in globule size with the passage of time, especially at higher temperatures while decreased at low temperatures. Centrifugation test did not cause phase separation till the 45th day, but little effects after 2 months. Chemical stability analysis by HPLC at the end of 3 months showed that ascorbyl palmitate and sodium ascorbyl phosphate were almost stable in all multiple emulsions with no influence of their location in a carrier system. Multiple emulsions were found a stable carrier for hydrophilic and lipophilic vitamin C derivatives to enhance their desired effects. Considering that many topical formulations contain simple vitamin C it is suggested that present study may contribute to the development of more stable formulations with a combination of vitamin C derivatives to enhance their cosmetic benefits.

Keywords: W/O/W multiple emulsion, ascorbyl palmitate, sodium ascorbyl phosphate, physical stability, chemical stability

Vitamin C performs as a free radical quencher that whitens the skin by mitigating melanin pigmentation and also mends skin elasticity by fostering collagen synthesis (1-3). The use of ascorbic acid has become limited in topical formulations because of its low chemical stability in heterogeneous systems (4). Fast oxidation and irreversible chemical transformation made ascorbic acid extremely reactive and unstable in dispersions. The use of less reactive derivatives like sodium ascorbyl phosphate salts or ascorbyl esters is a struggle to prolong their stability (5).

Sodium ascorbyl phosphate and ascorbyl palmitate are the most effective free radical quenchers, derivatives of ascorbic acid but differ in stability (6). Sodium ascorbyl phosphate (SAP) is a stable precursor of vitamin C that makes sure a constant delivery of vitamin C into the skin (7). Stability of ascorbyl esters in several colloidal carriers such as micro-emulsions, liposomes and solid lipid nanoparticles has been investigated. However, their long-term stability in such colloidal carries was still not adequate (5). Ascorbyl palmitate has a better sta-

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bility and capacity to penetrate into the skin than ascorbic acid (4). Due to its lipophilic character, ascorbyl palmitate penetrates more easily. Oxidation of ascorbyl palmitate by transition metal ions present in traces decreases its stability. Esterification of ascorbyl palmitate at 6 positions makes it less stable than other derivatives of ascorbic acid, including magnesium ascorbyl phosphate and sodium ascorbyl phosphate as this esterification does not prevent hydrolysis of the molecule (8). Structural properties of the formulations also influence the stability of all ascorbyl fatty acid esters (5).

Above mentioned two derivatives of vitamin C differ in their ability to permeate the skin because of their different hydrophilic/lipophilic properties. Sodium ascorbyl phosphate salts, being prodrugs, must be first converted by an enzymatic hydrolytic process before penetrating into the skin. But the lipophilic nature of ascorbyl palmitate makes its penetration easier as cell membrane is also lipophilic in nature (5).

Multiple emulsions are complex carrier systems where both oil in water (O/W) and water in oil (W/O) emulsions exist simultaneously (9). Multiple emulsions provide high entrapment efficiency, protection of active substance and controlled release of active substance (10). The current study investigated an alternative approach to use sodium ascorbyl phosphate in the aqueous phase and ascorbyl palmitate in the oily phase of a newly formulated multiple emulsion (W/O/W) to study the stability of resultant system and to attain their synergistic antioxidant effects of oxidative stress skin treatment.

EXPERIMENTAL

Materials

Ascorbyl palmitate (6-palmitoyl-L-ascorbic acid) and sodium ascorbyl phosphate (sodium L-ascorbyl-2-phosphate) from Sigma Aldrich, the lipophilic emulsifier - Cetyl Dimethicone copolyol (Franken, Germany), the hydrophilic emulsifier - polysorbate 80 (Merck, Germany), conductimetric tracer - $MgSO_4 \times 7H_2O$ (Merck, Germany), liquid paraffin (Merck, Germany).

Preparation of multiple emulsions

Multiple emulsions were developed using Cetyl Dimethicone copolyol as lipophilic emulsifier and polysorbate 80 as hydrophilic emulsifier. Different formulas using different concentrations of ascorbyl palmitate and sodium ascorbyl phosphate were tried for the selection of most stable W/O/W multiple emulsion. Composition of the tested emulsions is shown in Table 1.

The two-step emulsification procedure was adopted for the preparation of multiple emulsions (11). The primary W/O emulsion was prepared by emulsifying the oil phase with the aqueous phase in the presence of lipophilic surfactant. Both phases were preheated to 75°C in a digital water bath (Heidolph, Germany) before mixing. Mixing of W/O emulsion components was done using IKA Mixing Overhead Stirrer, Eurostar (IKA Werke, Germany) at 2000 rpm to obtain small inner droplets. In secondary emulsification step, W/O emulsion was dispersed in external aqueous phase

Table 1. Composition of the tested W/O/W emulsions.

Sample no.	F. code	Oil phase			Internal aqueous phase			External aqueous phase		
		Paraffin	CDC	Ascorbyl palmitate	Water	SAP	$MgSO_4$	Polysorbate 80	SAP	Water
1	ME_1	13.6%	2.4%	0.5%	63.54%	0.25%	0.56%	0.8%	0.25%	q.s
2	ME_2	13.6%	2.4%	0.5%	62.69%	0	0.56%	0.8%	0.5%	q.s
3	ME_3	13.6%	2.4%	0.5%	62.44%	0.5%	0.56%	0.8%	0	q.s
4	ME_4	13.6%	2.4%	0.5%	62.31%	0.38%	0.56%	0.8%	0.38%	q.s
5	ME_5	13.6%	2.4%	0.5%	62.69%	0	0.56%	0.8%	0.75%	q.s
6	ME_6	13.6%	2.4%	0.5%	61.94%	0.75%	0.56%	0.8%	0	q.s
7	ME_7	13.6%	2.4%	0.5%	61.94%	0.5%	0.56%	0.8%	0.5%	q.s
8	ME_8	13.6%	2.4%	0.5%	62.44%	0%	0.56%	0.8%	1%	q.s
9	ME_9	13.6%	2.4%	0.5%	62.0%	1%	0.56%	0.8%	0	q.s
10	ME_{10}	13.6%	2.4%	0.5%	61.57%	0.62%	0.56%	0.8%	0.62%	q.s

ME 1, 2 and 3 stands for multiple emulsion 1, 2 and 3 etc. CDC = cetyl dimethicone copolyol, SAP = sodium ascorbyl phosphate, $MgSO_4$ = magnesium sulfate, q.s = quantity sufficient.

containing a hydrophilic emulsifier to attain W/O/W multiple emulsion. This step was carried out under low speed (700 rpm) for 40 min to avoid rupturing of droplets. Formulated W/O/W emulsions were confirmed by microscopy.

Stability studies

All formulated multiple emulsions were stored at 40 ± 1°C for a period of 1 month. At the end of 1 month, all the multiple emulsions were subjected to centrifugation and checked for stability. The first three multiple emulsions (ME_1, ME_2 and ME_3) with 0.5% concentration of ascorbyl palmitate and sodium ascorbyl phosphate each, were found stable and were further subjected to physical and chemical evaluations for the period of 3 months. Selected emulsions were kept in the different storage conditions: i.e., room temperature (25 ± 1°C), low temperature (8 ± 1°C), high temperature (40 ± 1°C) and high temperature with humidity (40 ± 1°C with 75% relative humidity). Emulsions were then evaluated for rheology, microscopic analysis, conductivity, pH, centrifugation, liquefaction and color at baseline and on day 7, 15, 30, 45, 60 and 90. Chemical stability evaluation was done by high performance liquid chromatography (HPLC). Before any measurement, temperature of each emulsion sample was allowed to reach 25°C.

Physical stability analysis

The viscosities of the ME_1, ME_2 and ME_3 emulsions were determined at 25 ± 1°C in a Brookfield programmable rheometer (Model DV.III; Brookfield Engineering Laboratories Inc., USA) with spindle CP41 having speed ranging from 100 to 200 rpm. Rheocalc V 2.6 (Microsoft Corporation) software was used as a support program to produce the results of rheological behavior. Each time 0.5 g of emulsion sample was taken for viscosity measurement. The obtained results of viscosity measurements were fitted to the Power Law, which is:

$$\tau = kD^n$$

where τ is the shear stress, D is shear rate, k is consistency index, and n is a flow behavior index. Consistency index k has relation with apparent viscosity. Flow behavior index n determines the degree of non-newtonian behavior.

Microscopic analysis of ME_1, ME_2 and ME_3 was carried out under the microscope (Nikon E200, Nikon, Japan) with a camera (DCM-35 USB 2.0 and Minisee image software) to confirm multiple characteristics. After the dilution of multiple emulsions, observations were made at 100× magnification under oil emersion lens. Measurement of droplet size was done by using an ocular micrometer where stage micrometer was used for calibration.

Conductivity analysis of ME_1, ME_2 and ME_3 was performed to examine the release of the electrolyte initially entrapped in the internal water phase. The specific conductivity of the emulsions was measured directly using a Digital Microprocessor Conductivity Meter (WTW-Tetracon, Germany) at 25 ± 1°C.

The pH of ME_1, ME_2 and ME_3 was determined using a digital pH meter ProfiLine pH 197 (WTW, Germany). Centrifugation was carried out in 25 ± 1°C using centrifugal machine (Hettich EBA 20, Germany) at 5000 rpm speed for 10 min.

Chemical stability analysis by HPLC method

High performance liquid chromatography (HPLC) method was used for quantitative analysis of active compounds (ascorbyl palmitate and sodium ascorbyl phosphate) in ME_1, ME_2 and ME_3. Before injection of the sample into the HPLC system, extraction procedure was adopted from the method used by Polona spiclin in 2003 for the isolation of active ingredients from excipients. In this method sodium ascorbyl phosphate was extracted by taking 100 µL of sample which was diluted 1 : 100 (v/v) with tetrahydrofuran - 0.3 M phosphate buffer pH 4 (7 : 3) and then further diluted with 0.3M phosphate buffer pH 4 to a final 1 : 1000 (v/v) dilution and also ascorbyl palmitate was extracted by taking 100 µL of sample, which was diluted 1 : 100 (v/v) with methanol (12).

A standard curve was constructed to anticipate the percentage of active ingredients in different dosage forms. For standard curve 50, 100 and 1000 µg/mL dilutions of standard pure form of drug were prepared. By using standard curve parameters (intercept and slope), percentage of sodium ascorbyl phosphate and ascorbyl palmitate were calculated. Analysis was performed by using high performance liquid chromatography, equipped with a pump (Agilent 1200 series) and a variable wavelength detector (VWD, UV detector, Agilent USA). A column used was Hypersil BDS C_{18} (5 µm, 4.6 × 250 mm) for separation of the sample. The mobile phase was pumped at a rate of 0.8 mL/min. The sample amount injected into the system was 20 µL, with a run time of 12 min. pH of the mobile phase (5.8) was adjusted by using ortho-phosphoric acid. The λ max was obtained by the identification of response of different dilutions using a range of wavelength from 200 to 800 nm and finally wavelength at which all the dilutions of active drug showed maximum

response at 260 nm was identified. Evaluation of retention time for pure drug and samples was performed for the identification of drug. The retention time of active compounds was 9.4 min. At this stage variation of peaks at different times and concentrations was evaluated.

Statistical evaluation

Results of stability studies were subjected to statistical analysis by SPSS 20 software on the computer. One-way ANOVA test at the 5% level of confidence was applied to check the variation in results at different time and storage conditions.

RESULTS

Ten formulations with different concentrations (Table 1) of ascorbyl palmitate and sodium ascorbyl phosphate were evaluated and three of formulations (ME_1, ME_2 and ME_3) with 0.5% concentrations were found stable after centrifugation at accelerated temperature (40°C) and selected for further physical and chemical stabilities for the period of 3 months.

Results for change in viscosity with respect to shear rate for freshly prepared multiple emulsions (ME_1, ME_2 and ME_3) are shown in Figure 2. Viscosities of all multiple emulsions were found to decrease on increasing shear rates. Data on flow index and consistency index for ME_1, ME_2 and ME_3 at baseline and 3 months after preparation are given in Table 2.

Photomicrographs of ME_1, ME_2 and ME_3 are shown in Figure 3. Globule size measurements for freshly prepared ME_1, ME_2 and ME_3 and at different storage conditions after the period of 3 months are given in Table 3. A decrease in globule size was observed for samples kept at 8 ± 1°C and 25 ± 1°C while globule size of emulsion samples kept at 40 ± 1°C and 40 ± 1°C with 75% relative humidity was increased. Conductivity values of freshly prepared ME_1, ME_2 and ME_3 and at different storage conditions over the period of 3 months are given in Table 4. Conductivity values of ME_1, ME_2 and ME_3 increased continuously with the passage of time with little variations at 40 ± 1°C with 75% relative humidity. pH values of freshly prepared ME_1, ME_2 and ME_3 are given in Table 5. A decrease in pH values was observed with the passage of time. Phase separation, liquefaction and color of ME_1, ME_2 and ME_3 at baseline and at different time intervals kept at different storage conditions for a period of 3

Table 2. Results of rheological analysis of ME1, ME2 and ME3 fresh sample and samples stored at different storage conditions after 90 days.

ME_1	Fresh	After 3 months			
Temperature		8°C	25°C	40°C	40°C + 75%RH
Flow index	0.51	0.52	0.54	0.66	0.69
Consistency index	558.9	549.2	1246	529.4	549.2
ME_2					
Temperature		8°C	25°C	40°°C	40°C + 75%RH
Flow index	0.56	0.61	0.77	0.88	0.90
Consistency index	596.8	590.5	377.1	165.2	172.0
ME_3					
Temperature		8°C	25°C	40°C	40°C + 75%RH
Flow index	0.70	0.54	0.63	0.70	0.67
Consistency index	426.7	549.5	651.8	741.5	665.3

Table 3. Mean droplet size (μm) of ME1, ME2 and ME3 multiple emulsions.

Temperature	ME_1	ME_2	ME_3
Fresh	8.5 ± 2.33	8.9 ± 2.13	9.3 ± 2.13
8°C	4.8 ± 2.26	4.4 ± 7.50	5.5 ± 7.50
25°C	5.9 ± 7.55	4.4 ± 2.85	6.1 ± 2.85
40°C	8.0 ± 6.27	7.8 ± 5.04	7. 7 ± 2.85
40°C +75% RH	11.8 ± 4.25	12.3 ± 5.91	13.4 ± 5.99

Mean ± SD, RH = 75% relative humidity, (for multiple droplets n = 10).

Table 4. Conductivity values (S/m) of ME_1, ME_2 and ME_3.

Time	8°C			25°C			40°C			40°C with 75% RH		
	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3
Fresh	14.2	14.5	17.7	14.2	14.5	17.7	14.2	14.5	17.7	14.2	14.5	17.7
7 days	14.4	18.2	21.9	15.5	17.2	21.9	16.2	19.9	27.3	14.9	19.4	14.8
15 days	21.5	20.4	20.3	15.9	18.2	22.0	18.5	19.9	34.5	14.9	21.2	12.3
30 days	28.9	29.3	21.0	21.5	24.7	23.0	19.1	24.3	31.8	18.5	27.4	12.3
45 days	34.1	38.5	23.0	21.5	25.7	23.7	20.1	24.8	36.4	18.7	12.0	9.7
60 days	35.8	48.7	23.6	32.5	29.8	29.3	22.3	26.7	39.8	20.3	10.11	9.3
90 days	51.1	53.7	23.9	34.3	44.7	34.1	25.3	31.3	34.5	25.3	10.0	7.5

Table 5. pH values of ME_1, ME_2 and ME_3.

Time	8°C			25°C			40°C			40°C with 75% RH		
	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3	ME_1	ME_2	ME_3
Fresh	6.50	6.60	6.45	6.50	6.60	6.45	6.50	6.60	6.45	6.50	6.60	6.45
7 days	6.16	6.19	6.26	6.30	6.12	5.72	5.85	5.95	5.92	5.93	6.17	5.65
15 days	6.03	6.07	5.97	6.31	6.12	5.66	5.80	5.89	5.77	5.88	6.13	5.61
30 days	5.86	5.99	5.85	5.71	5.95	5.63	5.69	5.80	5.62	5.69	5.91	5.51
45 days	5.73	5.95	5.67	5.71	5.90	5.60	5.50	5.76	5.60	5.59	5.89	5.50
60 days	5.56	5.9	5.62	5.59	5.89	5.57	5.50	5.66	5.57	5.55	5.70	5.49
90 days	5.48	5.68	5.59	5.50	5.62	5.49	5.49	5.47	5.59	5.49	5.60	5.49

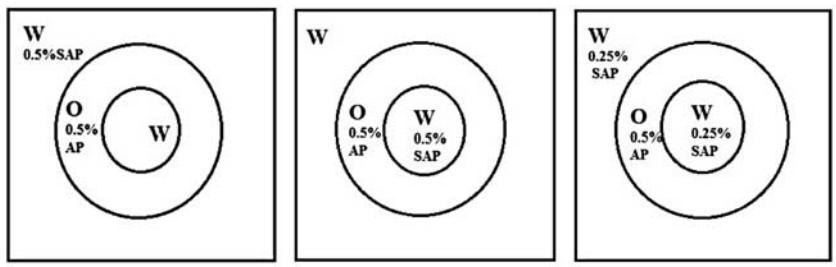

Figure 1. Layout diagrams of ME_1, ME_2 and ME_3

months are summarized in Table 6. Slight change in color, liquefaction and phase separation was observed throughout the study period.

Quantitative analysis of formulations by HPLC method is given in Table 7. Slight degradation of active compounds at the end of 3 months showed their stability in multiple emulsions.

DISCUSSION

Several multiple emulsions using different concentrations of sodium ascorbyl phosphate and ascorbyl palmitate as functional ingredients to attain the synergistic antioxidant effects were developed (Table 1). Emulsifiers used were Cetyl Dimethicone copolyol as lipophilic emulsifier for oil phase and polysorbate 80 as a hydrophilic emulsifier for aqueous phase. Concentration of emulsifiers was the same in all multiple emulsions. Paraffin oil was used as oil phase and $MgSO_4$ as an electrolyte. Formulated emulsions were placed in stability chamber at 40 ± 1°C (accelerated condition) for a period of 1 month. Among those, three emulsions (ME_1, ME_2 and ME_3) with 0.5% concentration of

Table 6. Physical characteristics of ME_1, ME_2 and ME_3.

	Parameters	Fresh			7 days			15 day			30 days			45 days			60 days			90 days		
		M E_1	M E_2	M E_3	M E_1	M E_2	M E_3	M E_1	M E_2	M E_3	M E_1	M E_2	M E_3	M E_1	M E_2	M E_3	M E_1	M E_2	M E_3	M E_1	M E_2	M E_3
A	Color	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW
	Liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Phase separation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	Color	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	YW	YW	SYW	YW	YW	SYW
	Liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	++	+	+	++
	Phase separation	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	++	+	+	++
C	Color	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	SYW	YW	YW	SYW	YW	YW	SYW	YW	YW	SYW
	Liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	++	++	++	++
	Phase separation	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	++	++	++	++
D	Color	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	OW	SYW	YW	YW	SYW	YW	YW	SYW	YW	YW	SYW
	Liquefaction	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	++	++	++	++
	Phase separation	-	-	-	-	-	-	-	-	-	-	-	-	+	+	++	++	++	++	++	++	++

Where, A = 8°C, B = 25°C, C = 40°C, D = 40°C with 75% RH. ME_1 = multiple emulsion 1, ME_2 = multiple emulsion 2, ME_3 = multiple emulsion 3. OW = off white, YW = yellowish white, SYW = soft yellowish white, – = no, + = little, ++ = more.

sodium ascorbyl phosphate and ascorbyl palmitate each were found stable and subjected to physical and chemical stability analysis for a next observational period of 3 months. The reason behind the stability of above selected emulsions may be that at 0.5% concentration, ascorbyl palmitate and sodium ascorbyl phosphate were less exposed to environmental oxygen thus chances of degradation were smaller. Oyetade et al. reported that a significant negative correlation exists between ascorbic acid and time of storage and exposure to air (13). In selected formulations, small amount of ascorbyl palmitate and sodium ascorbyl phosphate made their exposure to air minimal. ME_1 contained 0.5% ascorbyl palmitate in oil phase and 0.25% sodium ascorbyl phosphate in internal and external water phase of water-in-oil-in-water emulsion (Fig. 1A). As ME_1 contained a very small amount of SAP in outer aqueous phase, so its chances of exposure to environmental oxygen and consequently degradation were lower. ME_2 contained 0.5% ascorbyl palmitate in oil phase and 0.5% sodium ascorbyl phosphate in external water phase of water-in-oil-in-water emulsion (Fig. 1B). Small amount of above both derivatives in oil and external aqueous phase made it also stable. ME_3 contained 0.5% ascorbyl palmitate in oil phase and 0.5% sodium ascorbyl phosphate in internal water phase of water-in-oil-in-water emulsion (Fig. 1C). As ME_3 contained no vitamin C derivative in external aqueous but a small amount in oil phase and internal aqueous phase so it was also stable.

Selected emulsions were again formulated and their long term stability in terms of rheology, microscopy, conductivity, pH, centrifugation and HPLC analysis was done. The study of rheological behavior of formulations during the development process is essential as such studies provide behavior of the product during use. The viscosities of ME_1, ME_2 and ME_3 were found to decrease on increasing shear rates (Fig. 2) which showed their shear thinning behavior. At low shear rate, the aggregates may be deformed, while at high shear rate, the aggregates may be broken down to individual globules, decreasing viscosity. The decrease in viscosity was higher at 40 ± 1°C and 40 ± 1°C with 75% relative humidity. There may be two reasons behind this phenomenon. At higher temperatures there may be: a) diffusion of water molecules from the internal aqueous phase to the external aqueous phase, b) bursting of multiple globules due to osmotic pressure (14). Power law analysis of ME_1, ME_2 and ME_3 showed pseudo-plastic behavior, with a flow index below 1 (Table 2). Pseudo-plasticity is a desirable rheological property in cosmetic formulations as it improves application and spreading, providing a pleasant sensory feeling. Newtonian materials do not behave in this way, because when applied to the skin they run very quickly (15).

One-way ANOVA statistical test at the 5% level of significance was applied to check any deviation in viscosity of ME_1, ME_2 and ME_3 in different conditions of storage. Statistically significant differences were observed as the p-value was 0.0001 (< 0.05).

Microscopic analysis is essential for understanding the characteristics of multiple emulsions (16). Results of current investigation confirmed the formation of multiple emulsions as photomicrographs showed multiple droplets with small inner droplets inside (Fig. 3). The size of globules is an important parameter that determines the stability of emulsions (17). Globule size of fresh samples of ME_1, ME_2 and ME_3 was 8.5 ± 2.33, 8.9 ± 2.13 and 9.3 ± 2.13 µm, respectively (Table 3). After 3 months, a significant decrease in globule size was observed at 8 ± 1°C and 25 ± 1°C. The decrease in size may be due to shrinkage of globules resulting from the expulsion of internal water droplets towards external water phase. However, at 40 ± 1°C and 40 ± 1°C with 75%RH, an increase in globule size was observed. The increase in globule size may be due to the coalescence of globules which showed

Table 7. HPLC analysis of ME_1, ME_2 and ME_3.

	Ascorbyl palmitate			Sodium ascorbyl phosphate		
F. code	Theoretical concentration (mg)	Obtained concentration (mg)	%age	Theoretical concentration (mg)	Obtained concentration (mg)	%age
ME_1	500	445.6	89.1	500	477.8	95.6
ME_2	500	434.3	86.9	500	456.2	91.3
ME_3	500	455.8	91.2	500	467.5	93.5

mg = milligram

instability of emulsions under accelerated conditions (18). One-way ANOVA statistical test at the 5% level of significance was applied to check any deviation in globule size of ME_1, ME_2 and ME3 in different conditions of storage. Statistically non-significant differences ($p > 0.05$) were observed regarding differences in globule sizes.

Conductivity measurement provides the evidence about the entrapment of active substances in the primary emulsion (W/O) of W/O/W emulsion. The release of active substances is linked to amount of electrolyte which is incorporated in the internal aqueous phase of primary emulsion. More electrolytes are released; more the active substance will release from internal aqueous phase to external aqueous thus, less sustained will be the effects (19). The conductivity value of ME_1, ME_2 and ME_3 were found to increase continuously with the passage of time at 8 ± 1°C, 25 ± 1°C and 40 ± 1°C (Table 4). It is believed that during storage, conductivity values increase, which may be due to: diffusion of electrolyte, the coalescence of internal and aqueous phases, destruction of oil film because of osmotic pressure and the leakage of internal aqueous phase. However, at 40 ± 1°C with 75% relative humidity, ME_2 and ME_3 showed a decrease in conductivity values. This decrease in conductivity values may be ascribed to phase separation and extended larger droplet radii (20).

The pH is an important parameter regarding the effectiveness and stability emulsions (22). pH of the skin ranges between 4 to 6.5 and 5.5 is considered to be average pH of the skin where dry skin is not only dry, but too high in alkaline (20). pH of freshly prepared ME_1, ME_2 and ME_3 was found to be 6.50, 6.60 and 6.45, respectively. With the passage of time, a decrease in pH was observed in all multiple emulsions (Table 5) which continued till 3 months but this variation was within the skin pH range. This decrease in pH might be due to diffusion of water (pH 5-7) from internal to external phase or due to production of highly acidic by-products as

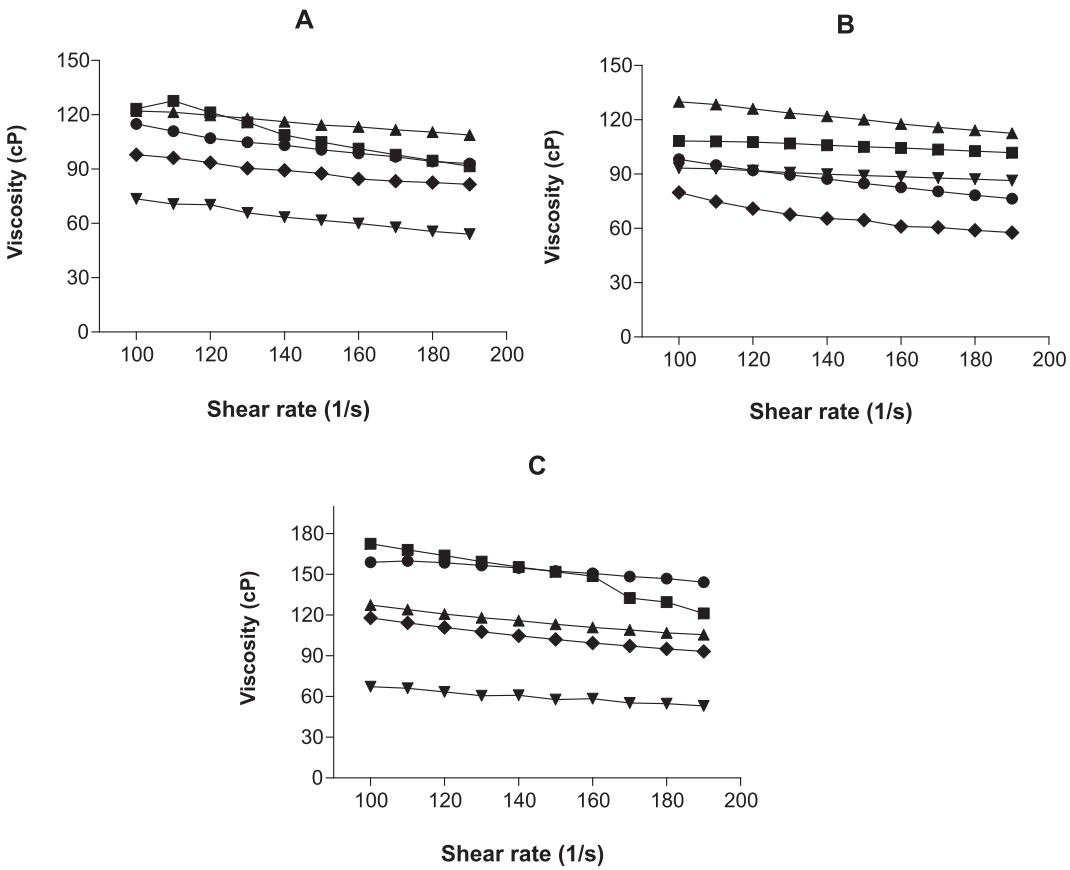

Figure 2. Viscosities of fresh multiple emulsion samples and at different storage conditions on varying shear rate. Alterations in viscosity for (**A**) Multiple emulsion ME_1, (**B**) Multiple emulsion ME_2, (**C**) Multiple emulsion ME_3

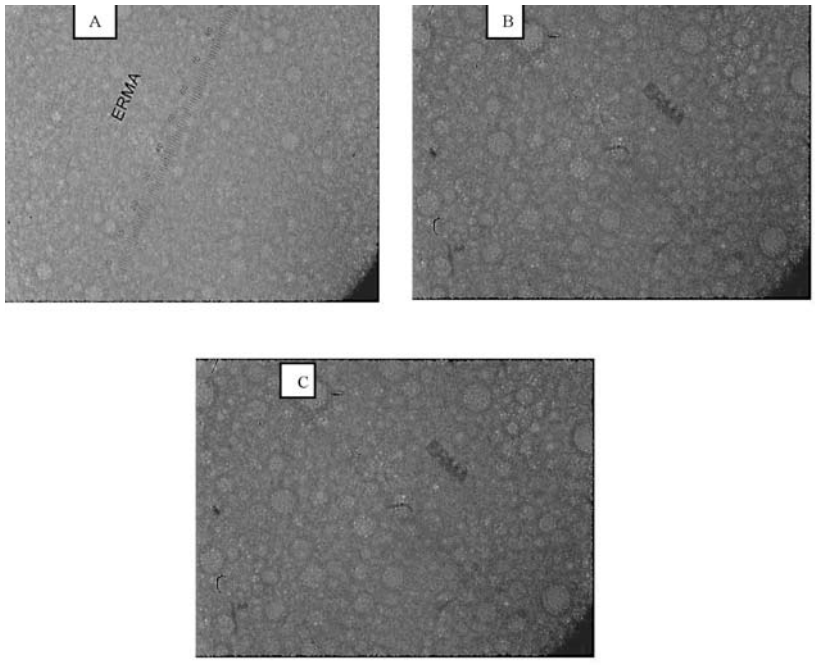

Figure 3. Photomicrographs of multiple emulsions immediately after preparation; (A) ME_1, (B) ME_2, (C) ME_3

reported (11) from any of the ingredients in the oil phase.

The centrifugation test is a useful tool for assessing and predicting the shelf life of emulsions and is based on the principle that centrifugal force separate two or more substances of varied densities, such as two different liquids or a liquid and a solid (21). No phase separation on centrifugation was observed in samples of ME_1, ME_2 and ME_3 kept at 8 ± 1°C up to 3 months. A slight phase separation, however, recorded in the samples kept at 25 ± 1°C, 40 ± 1°C and 40 ± 1°C with 75%RH which continued throughout the study period of 3 months (Table 6). This indicated that emulsion samples were almost stable at 8°C. This was presumably due to the proper homogenization speed kept during emulsion formulation, which might have reduced the globule size and prevented the breakage of the emulations during testing procedures (22). However, under accelerated storage conditions, emulsions were in stress, so the phase separation was observed in all emulsion samples stored under these conditions. This phase separation test predicts the stability of multiple emulsions (23).

Liquefaction in emulsions is attributed to the passage of water from the internal phase to external phase and is a sign of instability (21). In our study, no liquefaction was observed in the ME_1, ME_2 and ME_3 samples kept at 8 and 25°C throughout the study period of 3 months. However, slight liquefaction was observed in the samples kept at 40 ± 1°C and 40 ± 1°C with 75% RH after the 45th day, which continued till 3 months (Table 6). The absence of liquefaction at low and room temperatures provided the evidence for the stability of emulsions under study, while at 40 ± 1°C and 40 ± 1°C with 75% RH, liquefaction might be due to temperature effects as high temperature contributes to decrease in viscosity which results in liquefaction (24).

The color of freshly prepared ME_1, ME_2 and ME_3 was off-white. Emulsions stored at 8°C maintained their color for 3 months. While samples of ME_1, ME_2 and ME_3 stored at 25 ± 1°C, 40 ± 1°C and 40 ±1°C +75% RH showed few changes in color, which was marked by the following colors: yellowish white and soft yellowish white (Table 6). For example, for ME_1 and ME_2, the change in color was yellowish white which appeared from the 45th day at higher temperatures and persisted up to the 3 months of the study period. For ME_3, the change in color was soft yellowish white which appeared from the 30th day and persisted up to the 3 months of the study period. This change in color may be due to little degradation of active compounds as during observation emulsions were exposed to light. Moreover, change in color may be presumably due to oily phase separation promoted at higher temperatures (23).

Chemical stability analysis by HPLC showed little degradation of active compounds at the end of 3 months. Stability of ascorbyl palmitate and hydrophilic sodium ascorbyl phosphate was tested in all the three multiple emulsions, stored at 25°C at 0.5% initial concentration of each compound. As expected, both compounds were found stable in all the three multiple emulsions with no influence of their location in the carrying system. After 3 months, more than 90% of non-degraded sodium ascorbyl phosphate and more than 85% of non-degraded ascorbyl palmitate remained in all three multiple emulsions (Table 7). Literature data indicate that sodium ascorbyl phosphate is the most stable ascorbic acid derivative because of its chemical structure. In the structure of sodium ascorbyl phosphate, phosphate group is located in the second position on the cyclic ring which protects the enediol system of the molecule against oxidation, so its salts cannot act as an antioxidant agent to stabilize formulations (12). It has to be first converted into free vitamin C by enzymes present in the skin to achieve an anti-oxidizing function, which thus supports cutaneous antioxidant systems. The stability of the hydrophilic sodium ascorbyl phosphate was compared to that of lipophilic ascorbyl palmitate in all multiple emulsions at the same concentrations. In ascorbyl palmitate, palmitic acid is introduced in the sixth position of the cyclic ring so, the enediol system of the molecule is not protected (4). The fractions of non-degraded ascorbyl palmitate and sodium ascorbyl phosphate remaining after 3 months of storage are shown in Table 7. In all the three multiple emulsions, the stability of ascorbyl palmitate was somewhat lower than that of sodium ascorbyl phosphate. The obtained results confirm the stability of sodium ascorbyl phosphate and ascorbyl palmitate in multiple emulsions and support their possible use as functional ingredients in single cosmetic formulation to attain synergistic antioxidant action in the skin.

CONCLUSION

Lipophilic ascorbyl palmitate and hydrophilic sodium ascorbyl phosphate were incorporated in W/O/W type multiple emulsions, differing in their quantitative composition and location of active compounds in the carrier system. Three finally stable multiple emulsions (ME_1, ME_2 and ME_3) with ascorbyl palmitate and sodium ascorbyl phosphate in 0.5% concentration of each were subjected to physical and chemical characterization. Physical stability analysis in term of rheology, microscopy, conductivity, pH and centrifugation showed acceptable stability of ME_1, ME_2 and ME_3 irrespective of location of their active compounds in the carrier system. Addition of ascorbyl palmitate and sodium ascorbyl phosphate in combination have no substantial effect on the stability of ME_1, ME_2 and ME_3 except at accelerated conditions. Chemical stability analysis by HPLC also showed slight degradation of active compounds at the end of 3 months. Multiple emulsion was found a stable carrier for hydro- and lipophilic vitamin C derivatives and present study may contribute to the development of more stable multiple emulsions with different combination of hydro- and liposoluble vitamin C derivatives to attain their synergistic antioxidant effects in cosmetic industry.

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Conflict of interest

Authors have no competitive interests for this manuscript.

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EFFECT OF POLYMER AND PLASTICIZER ON THIN POLYMERIC BUCCAL FILMS OF MELOXICAM DESIGNED BY USING CENTRAL COMPOSITE ROTATABLE DESIGN

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Abstract: Aim of the present work was to design fast dissolving buccal film of meloxicam using central composite rotatable design and to evaluate the effects of polymer and plasticizer on formulation and characterization of the buccal films. Meloxicam was incorporated in film as model drug, HPMC E15 was used as film forming agent and polyethylene glycol (PEG) 400 was used as plasticizer. Films were fabricated using solvent casting technique. Prepared films were subjected to study various evaluation parameters. Dissolution studies were carried out for 30 min, using phosphate buffer of pH 6.8. Drug-excipients compatibility was studied using Fourier transform infra-red spectroscopy (FTIR). X-ray diffractometry (X-RD) was used to observe the crystalline or amorphous nature of the drug. Differential scanning calorimetry was used for thermal analysis of the drug and films. Optical microscopy and scanning electron microscopy were used to study the surface morphology. Results revealed that apparently the films were of smooth surface with uniform mixing of drug and excipients. Folding fortitude was > 100 in all the formulations. Weight variations were in acceptable range. Moisture loss was directly linked with concentration of plasticizer. Although buccal films were showing rapid release of the drug but still it was noticed that increasing concentration of HPMC E15 was the cause of drug retardation as well as delay in the total dissolution time, while PEG 400 was facilitating the drug release from the formulated films. Formulation F5 released approximately 100% drug in 5 min. All formulations individually showed total dissolving time in the range of 48-120 s. There were no noticeable interactions between drug and excipients. Finally, it was concluded that meloxicam containing films can be optimized using statistical tools, and HPMC E15 in combination with PEG 400 as plasticizer can be effectively used in the films formulation.

Keywords: CCRD, buccal films, meloxicam, DSC, X-RD, HPMC E15

Purpose of current improvements in novel drug delivery systems is to enhance safety and usefulness of drugs by designing a dosage form that is appropriate for administration and superior in accomplishing patient compliance (1), Modification in the buccal drug delivery lead to the advancement of dosage form, from conventional compact dosage forms to modified release dosage forms to orally disintegrating tablets and lastly development of immediately disintegrating buccal films (2, 3). Various polymers are reported in the literature which are effectively used in buccal films, e.g., cellulose based polymers especially hydroxypropyl methylcellulose and its various grades like E4, E5, E6 and E15 etc. Meloxicam is the member of the oxicam family. It has poor water solubility and longer half-life of 15-20 h. Its molecular weight is 351.40072 g/mol. It is a yellow crystalline powder having melting point 242-250^{o}C. It is a non-steroidal anti-inflammatory drug with the potential of being effective in various pain and inflammatory disorders like arthritis, lower back ache, osteoarthritis, low back pain, in primary dysmenorrhea and fever etc. Meloxicam blocks cyclooxygenase (COX) synthesis (4). Longer half-life is one of the important properties of oxicam family, which make it possible to deliver the drug once a day. Normal dose of meloxicam is 7.5 mg per day. Considering these features of meloxicam, the attempt was made to develop fast dissolving buccal film for faster onset of

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action. Fast dissolving buccal films (FDBFs) were prepared using central composite rotatable design which is an effective tool for the optimization of formulation, as formerly established by Box and Wilson (5).

MATERIALS AND METHODS

Materials

Meloxicam was used as active pharmaceutical ingredient and was gifted by Pharmedic Laboratories Lahore Pakistan; HPMC E5 (film former - a low viscosity polymer and 2% solution of which showed viscosity of 12-18 cP) and polysuccralose used as sweetening agent were obtained from Moringa Pharmaceutical Pvt. Ltd., Lahore, Pakistan. Orange flavor used as flavoring agent, PEG 400 used as plasticizer and Tween 80 that was used as solubilizer, were taken from research laboratories of The University of Lahore, Pakistan.

Methods

Central composite rotatable design (CCRD) was used for the design and optimization of buccal film by Design Expert software (version 8.1). Two variable HPMC E15 (X1) and PEG 400 (X2) were selected. Nine out of 13 formulations were selected for the development of buccal films to deliver meloxicam. Design of formulation of meloxicam buccal films is described in Table 2.

Optimization data analysis and numerical optimization

Various responses were studies by using Design Expert Software (Version 7.1.6, Stat- Ease Inc., Minneapolis, MN). Polynomial equation, including interaction and quadratic factors were studied for all the response variables using multiple linear regression analysis (MLRA) approach. The mathematical form of the MLRA model is given as (6):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 - \beta_1^2 X_1^2 - \beta_2^2 X_2^2$$

where, βo is the intercept demonstrating the arithmetic mean of all numerical outcomes of 13 trials; β_1 and β_2 are the coefficients calculated from the observed experimental values of Y; and X_1 and X_2 are the coded levels of the independent variable(s). The terms X_1X_2 and Xi^2 (i = 1 to 2) represent the interaction and quadratic terms, respectively. ANOVA (provision in the Design Expert Software) was used for the statistical validation of the polynomial equation. Various possibility and grid explorations were conducted to find the composition of optimal formulations. Also, the 3-D response surface graphs and 2-D contour plots were created using the output files generated.

Arithmetical optimization using the desirability approach was employed to find the ideal sets of the formulation variables to achieve the anticipated response.

Values x_{max} and x_{min} were maximum and minimum values of x respectively; $\alpha = 2k/4$; k = number of variables (in this study; $\alpha = 13/4 = 1.682$)

Preparation of fast dissolving buccal films of meloxicam

Solvent evaporation method was the commonly used method for the preparation of thin films. The same method was adopted in the current study. Accurately weighed amount of drug was dissolved in the mixture of methanol and phosphate buffer of pH 6.8 to obtain the concentration of 7.5 mg/mL of

Table 1. Relation between coded and actual values of the factors.

-β	X_{min}
-1	$[(x_{max} + x_{min})/2] - [(x_{max} - x_{min})/2\alpha]$
0	$[(_{xmax} + x_{min})/2]$ +1 $[(x_{max} + x_{min})/2] + [(x_{max} - x_{min})/2\alpha]$
+β	X_{max}

Table 2. Independent variables and their different levels used in formulation of meloxicam films.

Name	Variables	Xmin	Xmax	-ß	-1	0	1	+ß
E5 mg	X1	115	175	102.574	115	145	175	187.426
PEG %	X2	25	40	27.9289	30	35	40	42.0711

Table 3. Composition of meloxicam films according to CCRD.

	E15	PEG	T80	PS
Formulations	mg	mg	mg	mg
F1	187.4	65.5	18.7	3.7
F2	115	46	11.5	2.3
F3	145	50.7	14.5	2.9
F4	145	61.0	14.5	2.9
F5	175	52.5	17.5	3.5
F6	115	34.5	11.5	2.3
F7	102.5	35.9	10.2	2.0
F8	145	40.5	14.5	2.9
F9	175	70	17.5	3.5

Table 4. Evaluation parameters of FDBFs containing meloxicam.

Formulation code	Average thickness [mm]	Average weight [mg]	Average pH	Moisture loss %	Drug contents %	Total dissolving time [min]
F1	0.063	72.30	6.2	3.42	101.2	1.41
F2	0.0836	75.23	5.9	3.76	101.4	1.56
F3	0.0783	57.17	6.8	4.55	108.7	1.2
F4	0.0713	67.93	6.8	6.44	94.8	1.21
F5	0.056	50.43	6.1	3.66	107.7	0.48
F6	0.064	61.78	6.3	5.02	107.1	1.15
F7	0.074	55.32	6.6	3.93	107.4	1.39
F8	0.0813	48.68	6.3	7.12	96.4	2.00
F9	0.0726	59.29	6.2	4.27	110.9	0.56

the drug. Three percent solution of HPMC E15, 2% solution of PEG 400, 2% solution of Tween 80 and 1% solution of sweetener were prepared using distilled water as a solvent. Magnetic stirrer was used for the mixing and preparation of solutions. Solution of HPMC E15 was taken in a beaker with continuous mixing with magnetic stirrer. Drug solution was taken in a separate beaker on magnetic stirrer. PEG 400 and Tween 80 solutions were added to drug solution one by one with continuous stirring. After complete mixing this solution was added to the polymer solution and finally sweetener and flavoring agents were mixed in it. The mixture was subjected to continuous stirring for 30 min to achieve uniform mixing. Finally, the solution was poured in an "O" Petri dish having surface area of 24 cm^2. Petri dish was placed in hot air oven for drying at 40^oC for 24 h. After drying; the film was peeled off with help of sharp cutting edge knife, packed in aluminum foil and stored in desiccator for further studies.

Evaluation of prepared films

Surface morphology study

Surface morphology was studies by using light microscopy and scanning electron microscopy (SEM)

Light microscopy

Light microscopy of films was done to observe the film structure at micro level. Small strip of each film was cut and placed over glass slide and then mounted over the stage of Optika microscope 4083B3, Italy, and structure was observed at 40× power lens.

Scanning electron microscopy

Scanning electron microscopy was performed to observe the surface morphology of the formulated films. Films were cut in the small pieces of 1×1 cm^2 dimensions and scanned at 1000× magnification.

Thickness

Digital micrometer with least count 0.0001 mm was used to analyze the uniformity of the thickness. Thickness was measured from three different locations of the strips of each formulation. Mean and standard deviation were calculated. Thickness uniformity is an important parameter to predict the uniformity of the dose, as area with lesser thickness may contain lesser amount of drug (7).

Folding fortitude

Resistance of a strip to break upon folding is called folding fortitude (F.F.). It is measured as the number of times a strip can be folded at the same point until a crack seemed at that point. Number of times it took to break was reported as F.F. (8).

Weight uniformity

Uniformity in the weight of strips is a vital parameter to predict the dose uniformity of formulations. Four strips of each formulation were weighed individually on digital electronic weighing balance previously set to zero. Results were recorded as the mean weight and standard deviation was calculated for each formulation (9).

pH determination

pH of the buccal cavity is 5.5-7.4. Buccal films are made to dissolve in oral cavity, so their pH should lie in this range, because very acidic or alkaline formulations may cause local mucosal irritation. One strip was selected from each batch for pH measurement. As the pH of the dry strip cannot be determined, the strip was first converted into the state to measure the pH easily. For this purpose, it was first dissolved in 2 mL of distilled water. pH was measured by 25CW microprocessor benchtop pH/mV meter, BANTE Instruments, China. The electrode of the pH meter was brought to the strip solution and after 10 min to stabilize the pH value, the pH readings were noted (10).

Percent moisture loss (%MC)

Four strips from each batch were taken and weighed accurately, and then were kept in hot air oven at 40°C for 48 h (so that constant weight was achieved). Percent moisture contents or percent

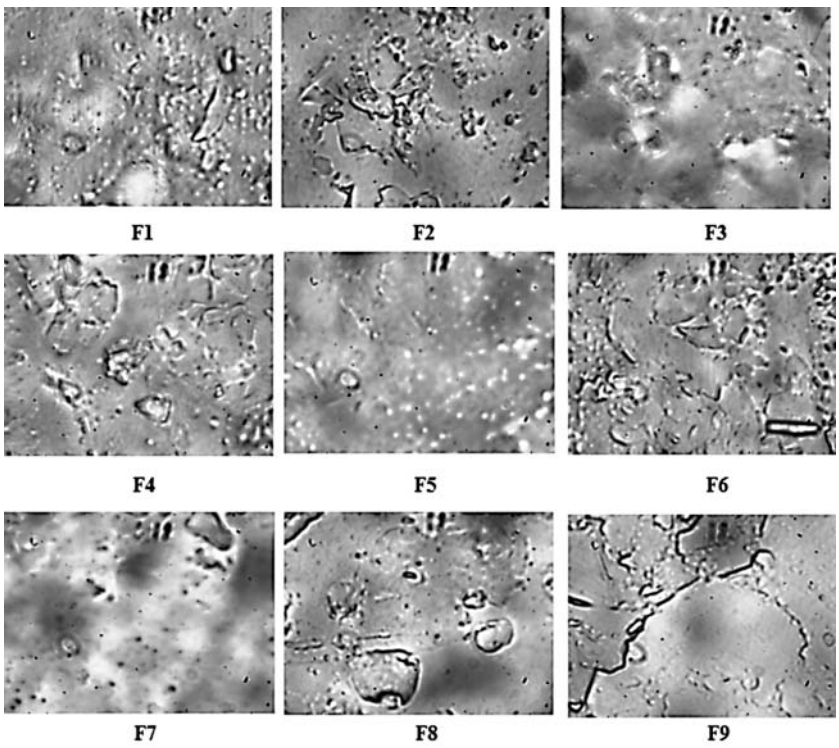

Figure 1. Light microscopy of HPMC E15 based FDBFs of meloxicam

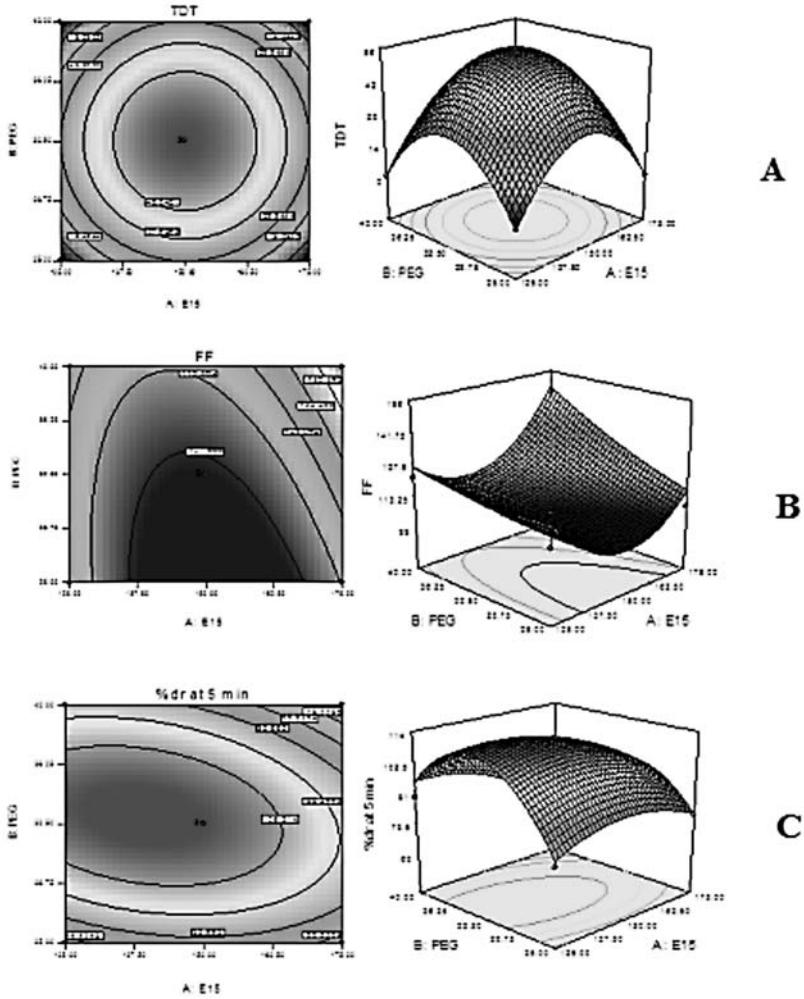

Figure 2. 2-D and 3-D plots of TDT (A), F.F. (B) and % drug release (C) of FDBFs of meloxicam

moisture loss was calculated according to the following equation:

$$\% \text{ Moisture Content} = \frac{Wo - Wd}{Wo}$$

where: Wo is an initial weight and Wd is a dried weight of the strip.

Total dissolving time (TDT)

In a Petri dish 10 mL of distilled water was added that was previously heated to 37°C. About 1 × 1 cm^2 area was cut from a strip. This strip was then carefully placed on the surface of water in such a way that the strip float over the surface and did not sink down or stick to the side walls of the Petri dish. Stop watch was started along placing the strip over water. The Petri dish was given slight swirling or shaking during counting the total dissolving time. Time when the strip was completely dissolved and became the part of the solution was taken as total dissolving time (11).

Uniformity of drug content

For content uniformity, individual film was dissolved in 100 mL phosphate buffer of pH 6.8. Two milliliters from this solution were diluted to 10 mL and absorbance was taken at 365 nm using double beam spectrophotometer (PG instrument T80) for calculation of % drug content.

***In vitro* drug release**

USP II dissolution apparatus (paddle type) was used for this purpose. Five hundred milliliters of phosphate buffer of pH 6.8 was used as dissolution medium. Apparatus was set at 37 ± 0.5°C and 50 rpm. Strip of 2 × 2 cm^2 area was cut from the films and carefully placed on the surface of glass slide

with the help of paper pin. Slide holding the strip was placed in the vessel. Five mL aliquots were drawn after every minute till 6th min and then at 8, 10 12, 16, 20 min and final sample was taken at 30 min. Absorbance of samples was measured at 365 nm using double beam spectrophotometer (PG instrument T80 Shimadzu, Japan) for calculation of percentage drug release (% DR).

Drug-excipients compatibility

FTIR studies were performed to find out any kind of drug-excipients interaction. To determine whether any type of chemical reaction is present between ingredients, FTIR was performed. ASTM standards for obtaining infra-red spectra for qualitative analysis of solids were followed for this study. According to ASTM standards alkali halide pressed pellet technique was used. ASTM recommends KBr for this purpose. Sample and KBr are to be mixed in 1/50 to 1/1000. The individual powder and selected film was filled in the die (approximately 10 mm) and 20 MPa pressure was applied; then, at this pressure. die was connected to vacuum pump for about 10 min to remove any moisture. FTIR spectra in the range of 400 to 4000 cm^{-1} were obtained for drug and excipients alone as well as for formulation (12).

Differential scanning calorimetry

Thermograms of meloxicam and film formulation were obtained by using differential scanning calorimeter (DSC-60A Thermal analyzer, Shimadzu, Japan). Samples of 7 mg were sealed in the aluminum pan and heated at the rate of 10°C/min from 35°C to 300°C. Nitrogen containing atmosphere was provided at the flow rate of 25 mL/min (13).

X-ray diffractometry

To study the change in the structure of meloxicam from crystalline to amorphous, the samples were analyzed by powder X-ray diffractometer. The experimental conditions were as follows: tube voltage was 45 kV, tube current 40 mA and scanning angle 2θ was 5–50° (13).

RESULTS AND DISCUSSION

Mathematical modeling

Mathematical relations created using multiple linear regression analysis for the calculated response variables are expressed as equations 1-3:

$$TDT = 56.00 + 0.14\ X1 + 0.038\ X2 - 0.035\ X1\ X2 - 27.57\ X1^2 - 27.13\ X2^2 \quad (1)$$

$$F.F. = 106.00 + 3.91\ X1 + 9.11\ X2 + 7.00\ X1\ X2 + 19.94\ X1^2 + 2.69\ X2^2 \quad (2)$$

$$\%\ DR\ (5\ min) = 111.84 - 5.90\ X1 + 0.25\ X2 - 5.88\ X1\ X2 - 7.17\ X1^2 - 20.96\ X2^2 \quad (3)$$

Light microscopy

Images obtained from light microscopy by Optika microscope at 40× magnification are shown in Figure 2. It can be seen that drug and polymer were distributed throughout the area. Few crakes and small pores and moisture droplets can also be observed in the figures of films.

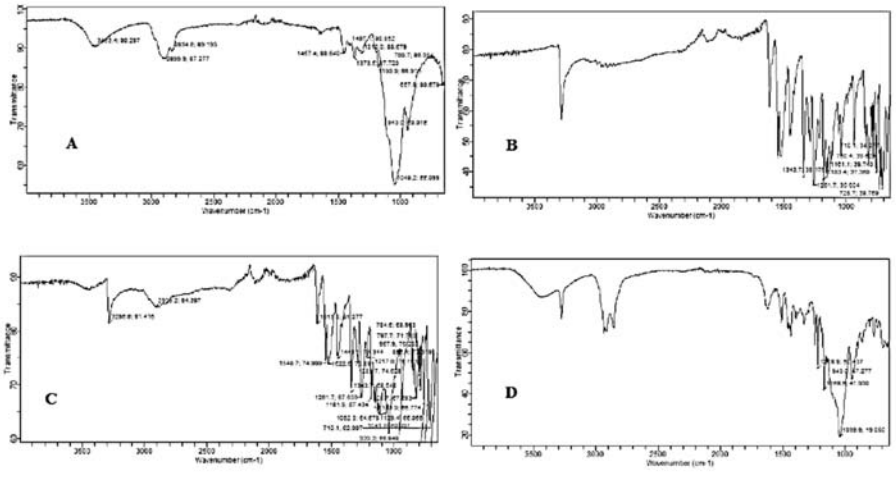

Figure 3. FTIR analysis of meloxicam (**A**), HPMC E15 (**B**), physical mixture of meloxicam and HPMC E15 (**C**) and FDBF containing meloxicam (**D**)

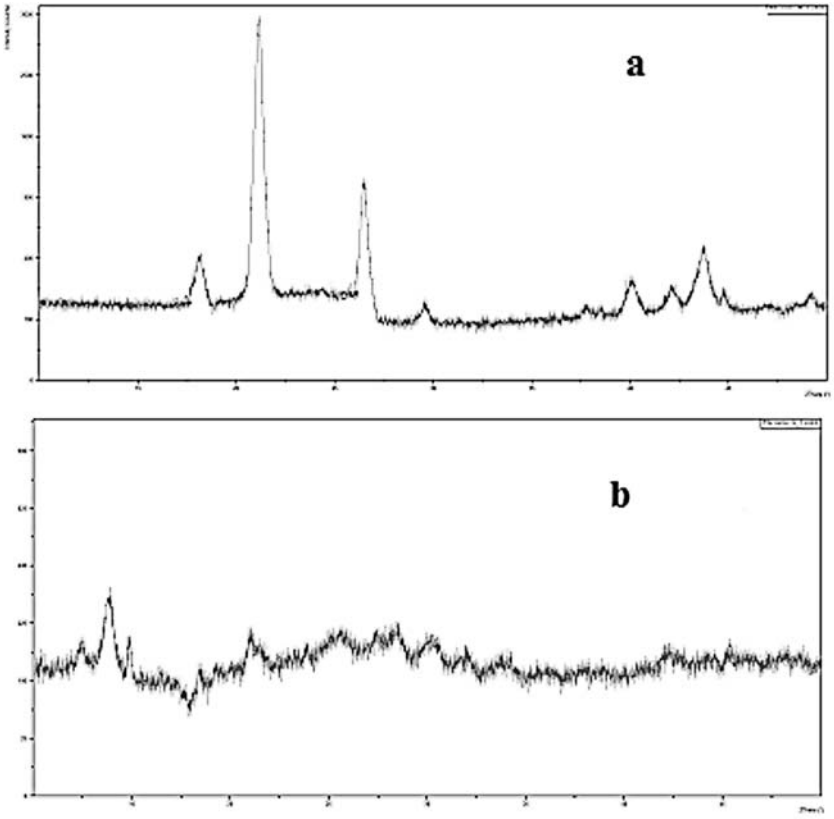

Figure 4. X-RD thermograms of meloxicam (**a**) and film formulation containing meloxicam (**b**)

Evaluation of meloxicam containing buccal films

Results were indicating the variation in thickness as well in weight variations test, attributed by the variation in the polymer's concentrations. Higher drug contents were the indication of good drug loading capacity of HPMC E15. Percent moisture loss studies described the ability of HPMC E15 to retain suitable amount of moisture. Total dissolving time was seemed to be affected by the varying concentration of polymer and plasticizer. Increased concentration of HPMC E15 was the major cause of delayed dissolving time.

Response surface analysis

3-D and 2-D counter plots were plotted for TDT, F.F. and % drug release to show the influence of both variables on studied characteristics.

Total dissolving time (response 1)

For TDT, $p < 0.0001$ suggested that model terms are significant, R-squared was found to be 0.9999. Adjusted R-squared was 0.9998 and predicted R-squared was 0.995.

3-D graph was shown in Figure 2A, expressing the influence of HPMC E15 and PEG 400. Polynomial equation (1) showed that increase in X1 (HPMC E15) can increase the TDT. X2 has comparatively less influence on enhancing the dissolving time, both interacting factor X1X2 have opposite effect, that means simultaneous variation in both PEG 400 and E15 enhance the dissolution of the films. Quadratic terms were also found to be decreasing the TDT. HPMC E15 has considerable impact on increasing the dissolving time. Polymer concentration usually has direct relationship with the thickness of the film and as the thickness increases, it causes a delay in dissolution rate of the film (8). From the results, it was concluded that an increase in polymer concentration, delayed the dissolving of the film (14).

Folding fortitude (response 2)

P value (0.0442) from ANOVA showed that model terms are significant for F.F. R-squared was 0.8938, Adjusted R-squared was 0.7611 and predicted R-squared values were 0.2451. RSM 3-D

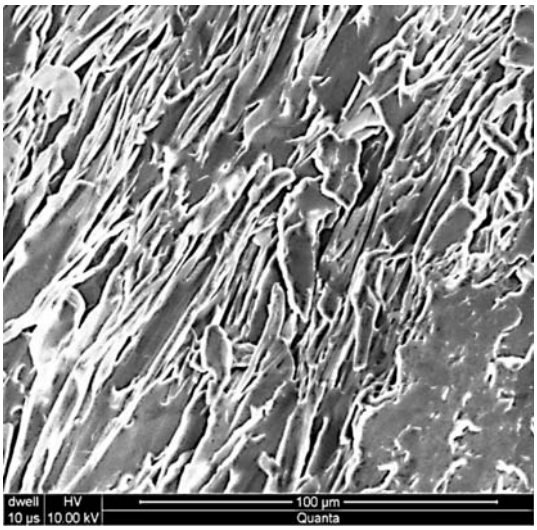

Figure 5. SEM of HPMC E15 based buccal film containing meloxicam

and 2-D counter plot are shown in Figure 2B, expressing the relationship of X1 and X2 and their effect on F.F. It is clear from the graphs that an increase in both variables; especially in X2 is increasing the F.F. value. From polynomial equation 2, it was even clearer that the mean of the dependent variable has a positive value, which suggested that the response is positive and both factors have a significant influence on the folding fortitude of the FDBFs of meloxicam. PEG 400 with greater values in equation (9.11) describes that it has pronounced effect on F.F. Plasticizer has the ability to improve the mechanical strength of the films. Increasing the concentration of PEG 400 enhances the mechanical properties of meloxicam loaded films and hence the F.F., since F.F. and mechanical properties are directly related to each other (7).

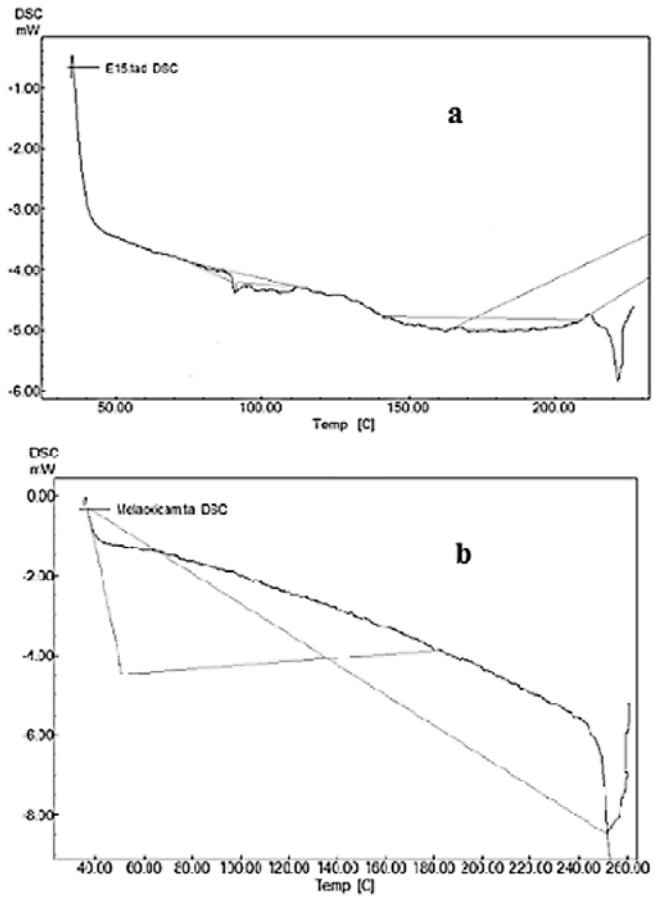

Figure 6. DSC thermogram of HPMC E15 based film containing meloxicam (**a**) and of pure meloxicam (**b**)

% Drug release (response 3)

Values of drug released from FDBFs after 5 min were taken (15) to analyze the response and effects of both variables. For % drug release response, p value (0.0081) suggested that model terms are significant; R-squared was found to be 0.9561. Adjusted R-squared was 0.9013 and predicted R-squared was 0.6880. Value of costant which was representing the mean of the variable, was a positive term (111.84). Results were indicating the influence of both X1 and X2 on the release of meloxicam from FDBFs. HPMC E15 is showing negative impact, that means that an increase in the concentration of polymer is decreasing the relase of the drug. The same can be observed in the 3-D plot of the response presented as figure 2 (C). PEG 400 showing positive effect and positive values are predicting that an increase in PEG 400 concentration will enhance the dissolution of meloxicam and hence, amount of the drug release. In the literature, it was found that PEG 400 is an effective co-solvent and it enhances the solubility as well as dissolution rate of the poorly water soluble drugs. Meloxicam is also a hydrophobic drug and PEG 400 can enhance the dissolution rate and solubility which may be the cause of greater drug release from the formulation containing greater concentration of PEG 400 (16).

Drug-excipients compatibility

FTIR of pure meloxicam, HPMC E15, their physical mixture and of formulated film was done. Scanning range was between 650-4000 cm^{-1} using FTIR Agilent Technology spectrometer. All the corresponding peaks showed same behavior in all three states. This suggested that there was no noticeable interaction between drug and excipients.

Meloxicam showed it principle absorption peak at 3290 cm^{-1}, which suggested the stretching of secondary amine (N-H). At 1620 cm^{-1} scissoring vibration of NH_2 and at 1540 cm^{-1} C=N stretch was observed. C-H wagging was seen at 1261.7 cm^{-1} and at 1161 cm^{-1}, S=O stretching appeared. Peak at 710 cm^{-1} described the rocking of C-H group. HPMC E15 FTIR spectra described O-H stretch at 3453.4 cm^{-1} while stretching of carboxylic acid group was seen at 2834.6 cm^{-1}. At 2899.9 cm^{-1} C-H stretch appeared. At 1457 cm^{-1} bending of C-H was seen. Peaks at 1190.9 cm^{-1} describe C-H wagging, at 1049 cm^{-1} showed C-N stretch and at 943 cm^{-1} describe the bending of O-H group. Physical mixture showed similar behavior at 3285.6 cm^{-1} (N-H stretch), 1615.8 cm^{-1} (NH_2 scissoring vibration) 1261.7 cm^{-1} (C-H wagging), 1548.7 cm^{-1} (C=N stretch), 1149.9 cm^{-1} (S=O stretch) and 710.1 cm^{-1} (C-H rocking).

X-ray diffractometry

X-RD studies showed that meloxicam was a semi-crystalline drug (17) as described in Figure 4a. Crystallinity of the meloxicam was decreased in the form of polymeric film (18), depicted in Figure 4b.

Scanning electron microscopy

Results of the SEM are described by Figure 5. Film surface was rough with cracks. It can be observed that drug is evenly distributed and entrapped by the polymer.

Differential scanning calorimetry

Thermal studies were conducted by using DSC for both pure meloxicam and meloxicam containing film formulations. DSC thermogram showed that there were no thermal interaction between the drug and excipients. A sharp peak at about 255^{o}C was obtained for meloxicam and similarly in the thermogram of meloxicam containing film formulation a sharp peak at about 230^{o}C was obtained. A little decrease in melting point may be attributed to the presence of plasticizer, because the plasticizer, like PEG, can decrease the transition temperature of the substances. Moreover, it may be due to conversion of crystalline meloxicam into amorphous form.

CONCLUSION

The present study was designed by using statistical tools. FDBFs were successfully developed with desirable characteristics. Effect of both polymer and plasticizer was observed. HPMC E15 was a suitable film forming agent and PEG 400 has excellent plasticizing abilities. It was concluded that statistical tools are valuable for the optimization of formulation, and combinations of selected film former and plasticizer were suitable for the development of FDBFs of meloxicam.

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Author (Muhammad Zaman) is very thankful to Dr. Farhan Saeed (Department of Polymer & Process Engineering, University of Engineering and Technology, Lahore, Pakistan) and Neyyar Talha (The University of Lahore) for their kind support and assistance in performing various characterization of prepared films.

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PHARMACOLOGY

THE POSOLOGY AND TROUGH CONCENTRATIONS OF DIGOXIN IN ADULT AND ELDERLY PATIENTS

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Abstract: Being a narrow therapeutic index drug, digoxin may cause harm if dosed without regular measurements of serum levels. Due to various limitations in its dosing, different challenges still exist in clinical practice. This study aimed to assess digoxin though concentrations after different regimens in adult and elderly patients, and to identify predictor variables for the ratio of given dose and digoxin trough level. This was prospective open-label study. Digoxin was administered *per os* as 0.125 or 0.25 mg during different continuous and interrupted dosage regimens. Study protocol allowed an additional therapy according to contemporary guidelines. Digoxin concentrations were determined using Abbott AxSYM Digoxin II assay in trough samples (1-3 per patient) after 3-4 weeks stable regimen. In total, 191 concentrations (104 patients) were analyzed. Digoxin weekly dose was in range 0.375-1.75 mg. On average, we observed slightly lower digoxin levels in HF patients. Results showed that in patients receiving digoxin with interrupted dosage regimen post-pause digoxin level was statistically significantly lower than pre-pause ($p < 0.05$). Based on multiple linear regression, the ratio of given dose and trough concentration was mainly predicted by clearance creatinine, and to lesser extent by patient's ideal body weight. Interrupted dosing schedule shows greater variability in drug levels comparing to continuous dosing, and it additionally causes difficulties in reaching and maintaining steady trough levels between doses. Hence, individualization of dosing regimen should be carefully guided based on target levels and not solely on clinical signs and symptoms.

Keywords: digoxin, dosing regimen, pharmacokinetics, therapeutic drug monitoring

Digoxin is indicated for the treatment of heart failure (HF) and atrial fibrillation (AF) (1-5). Due to the poor relationship between dose and therapeutic effect, narrow therapeutic index, and high pharmacokinetic variability, its dosing represents a challenge to the clinical practice (6, 7). Therefore, measurement of digoxin levels is recommended, and it is useful in individualizing therapy during the early stages of treatment, as well as for detecting non-adherence and risk for drug toxicity. Usual digoxin therapeutic range (0.8-2 ng/mL) has been challenged, especially in the management of HF. Hence, lower levels (0.5-0.8 ng/mL) showed 6.3% mortality reduction and hospitalizations in HF patients (8, 9). However, there are insufficient data regarding the appropriate digoxin targets. Nevertheless, it is reported that 0.8-1.2 ng/mL may be appropriate for AF (4). Digoxin concentrations > 2 ng/mL were frequently associated with drug's toxicity in almost all studies, while this limit is decreased to 1-1.2 ng/mL for HF patients (8-10). According to Goldberger et al., it is evident that different guidelines, textbooks, online resources, and clinical laboratories provide disparate digoxin therapeutic ranges (11). In order to achieve therapeutic levels, digoxin dosing is based upon patient-specific factors such as age, renal func-

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tion, and lean body weight. According to the recommendations, a majority of patients should benefit from daily dose of 0.125-0.25 mg, while in some patients a dosage of 0.0625 mg or 0.375 mg per day may suffice. As a result of distinct target concentrations for HF and AF, some guidelines suggest different dosing regimens as well: 0.0625-0.125 mg/day for HF and 0.125-0.25 mg/day for AF (4, 5, 12). In some health systems, digoxin oral form is available solely in 0.25 mg strength. Therefore, digoxin dosing requires 1/2 or even 1/4 of 0.25 mg tablet (13), and/or extending dosing interval from 24 to 48 h. In our clinical practice, like in others (14-17), digoxin dosing with a drug holiday of 1, 2 or 3 days a week was introduced in order to avoid drug toxicity. As therapeutic drug monitoring is not routine practice, interrupted dosing of digoxin is still present. Digoxin dosing 4 to 6 days a week is usually prescribed in elderly, patients with impaired renal function, or with a low lean body mass in order to minimize the possibility of digoxin toxicity (18). It is not clear whether this drug holiday is justified in all cases, since digoxin plasma levels might fall below therapeutic levels, and stable trough levels between doses are questionable. Hence, the purpose of our study was to describe different dosing regimens in the clinical settings, and to evaluate achieved digoxin trough levels. Additionally, we aimed to identify predictor variables for the ratio of given dose and digoxin trough level.

EXPERIMENTAL

This prospective study was conducted at the Clinic for Cardiology, Clinical Centre of University of Sarajevo, Bosnia and Herzegovina. Our study complies with Declaration of Helsinki, and it was approved by Institutional Review Board of Clinical Centre of University of Sarajevo. In total, 109 consecutive patients with AF and/or HF who were present at Clinic for Cardiology from September 2013 to March 2014 were considered for the study. The inclusion criteria were indication for digoxin, patients aged >18 years, who gave informed consent. Exclusion criteria were mainly related to treatment discontinuation due to/or digoxin intoxication, absolute or relative contraindication to digoxin including severe renal impairment when creatinine clearance (CLcr) < 15 mL/min, hypokalemia (< 3.5 mmol/L) or hypercalcemia (> 2.64 mmol/L) not correctable with medical treatments, pregnancy, severe dementia. CLcr was calculated using Cockroft-Gault equation based on total body weight (TBW) for patients with body mass index (BMI) < 25 kg/m^2, while for others using ideal body weight (IBW) (19). IBW was calculated from patient's height and gender.

Digoxin was available in form of 0.25 mg tablets (Lanibos®, Bosnalijek, Sarajevo, Bosnia and Herzegovina) (13). It was administered once a day or every other day with the same 0.125 or 0.25 mg dose (continuous dosing), or with different doses between days or variable dosing intervals between administered doses (interrupted dosing). Drug holiday periods were usually 1 or 2 inconsecutive days of the week. In the clinical settings, 0.25 mg digoxin tablets were split into halves using tablet cutter. The protocol allowed an additional therapy according to the contemporary guidelines for AF and HF. Compliance to treatment, before hospitalization, was assessed during regular medical visits by an interview with the attending physician. Data available for each patient included demographic characteristics (weight, height, age, gender), clinical data (diagnosis and history of disease, comorbidities, blood pressure, heart rate, ejection fraction, adverse events), laboratory data (serum creatinine, urea, serum transaminases, albumin, international normalized ratio, electrolytes (K^+, Na^+, Ca^{2+}) status), treatment information (dosing regimen, time of the last dose, concomitant therapy), schedule of blood sampling and digoxin serum concentrations. Digoxin dosing regimen was unchanged for at least 3-4 weeks before admitting patients to the cardiology ward, and thus sampling occurred while patients were hospitalized. One to three blood samples per patient were taken. Samples obtained from patients on continuous dosing without drug-free days were: prior to the morning dose ($trough_1$) and, in some patients, additional second pre-dose sample was taken ($trough_2$). In patients treated with interrupted regimen sampling scheme was corresponding to trough levels pre- and post-drug holiday, and in some patients where time between, at least two doses, was 48 h, additional sample related to 24 h post dose was available. Exact times of blood sampling were recorded.

Quantitative determination of digoxin was performed in the laboratory of the Clinical Centre using Abbott AxSYM Digoxin II assay. The imprecision coefficient of variation for the assay was less than 10%, and lower limit of quantification was 0.3 ng/mL (20). Descriptive and statistical data analyses were performed using software IBM SPSS Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY). The difference between mean trough levels in groups on 0.125 mg/24 h *versus* 0.125 mg/48 h was tested. Paired *t*-test was used to com-

pare trough concentrations on two time point occasions. Multiple linear regression analysis was performed to identify predictor variables and accompanying partial regression coefficients for the ratio of given dose and measured digoxin trough level. Statistical significance was defined when calculated probability value (p) was less than 0.05.

RESULTS

Based on study criteria, 5 patients were excluded due to toxicity: in 2 patients digoxin levels upon hospitalization, before stopping the treatment, were 5.8 and 6.9 ng/mL, while in others drug levels were < 2 ng/mL. Hence, in total 104 eligible patients were analyzed (Table 1).

Table 2 shows different dosage regimens identified in the clinical settings. Accordingly, digoxin weekly dose was in range from 0.375 to 1.75 mg, and drug was dosed every second day, 5, 6 or 7 days a week. In total, 191 digoxin concentrations were available for the analysis. Figure 1 shows distribution of measured digoxin trough levels in relation to dosing regimen and indication. On average, we observed slightly lower digoxin levels in patients with HF. None of the patients with HF had concentrations < 0.5 ng/mL or > 2 ng/mL, whereas in 59.37% of HF patients digoxin concentrations were > 1.2 ng/mL (Fig. 1). Furthermore, digoxin levels < 0.5 ng/mL were not observed in HF + AF patients as well. In 6 patients, when drug was dosed each day, measured trough concentrations were > 2 ng/mL, while in 4 patients on interrupted regimen measured trough concentrations pre-drug holiday were > 2 ng/mL (Fig. 1).

Demographic and clinical characteristics of patients were comparable between groups on 0.125 mg/24 h and 0.125 mg/48 h, hence, we compared concentrations prior to the next dose. The difference in the median values (as variances were unequal)

Table 1. Descriptive statistics of the study population.

Characteristic	Number (percent) / Mean ± standard deviation (range)
Gender (male)	34 (32.7%)
Age (years)	72.36 ± 10.59 (47 – 94)
Weight (kg)	80.38 ± 16.10 (50 – 135)
Height (cm)[a]	168.33 ± 9.00 (145 – 190)
Ideal body weight (kg)[a]	60.93 ± 9.25 (38.50 – 83)
Body mass index (kg/m^2) [a]	28.42 ± 5.32 (18.36 – 42.24)
Serum creatinine (μmol/L)	106.71 ± 37.30 (48 – 231)
Creatinine clearance (mL/min) [b]	49.31 ± 20.0 (14.89 – 113.46)
Heart failure	32 (30.8%)
Concomitant drugs	
ACE inhibitors	21 (20.80%)
Acetylsalicylic acid	43 (41.35%)
Amiodarone	23 (22.10%)
Anticoagulant drugs	65 (62.50%)
β-Blockers	36 (34.61%)
Calcium channel blockers (including verapamil, diltiazem)	22 (21.15%)
Furosemide	94 (90.38%)
Insulin and/or oral antidiabetic drugs	35 (33.65%)
Proton pumps inhibitors	49 (47.11%)
Spironolactone	56 (53.85%)
Statins	37 (35.58%)

[a]data from 18 patients were missing. [b]calculated according to Cockroft-Gault formula which included total or ideal body weight if body mass index was below or above 25 kg/m^2, respectively. It included 89 patients.

between the groups is greater than would be expected by chance (p = 0.01425). Hence, median (25-75 percentile) trough levels in group on digoxin 0.125 mg/24 h and 0.125 mg/48 h were 1.42 (1.36-1.60) and 1.02 (0.7-1.26) ng/mL, respectively.

A paired-samples *t*-test was conducted to test the hypothesis that trough digoxin concentrations pre-dose and 24 h or 48 h post-dose are equal. The first analysis tested trough digoxin concentrations on two occasions in patients on consistent doses between days and/or the same dosing interval between each dose. The estimated correlation between two time points was significant. However, according to the results, the mean differences between two trough levels in the same patient was not significant (p > 0.05), so the null hypothesis was

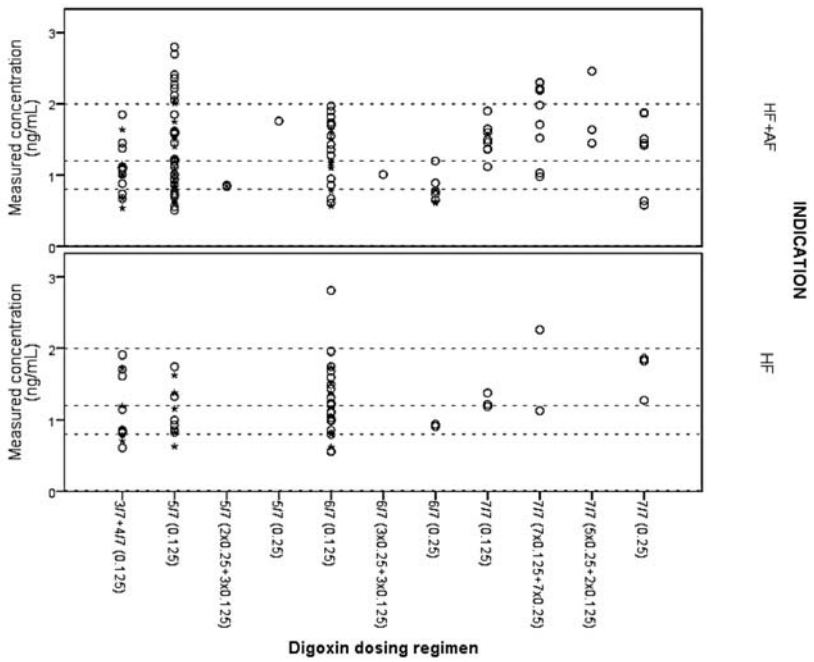

Figure 1. Measured trough digoxin concentrations in patients with heart failure (HF) and atrial fibrillation (AF) (upper) and HF (lower) *versus* digoxin dosing regimen. Trough levels pre-dose or pre-pause (open circle), and post-pause (star). Reference lines: y = 0.8, y = 1.2, y = 2 ng/mL

Table 2. Digoxin dosing regimens in patients included in the study.

Posology dose (mg) / (days of treatment/week)	Comment	Number of patients (%)
0.25 (7/7)	the same dose each day	9 (8.65%)
0.125 (7/7)		10 (9.61%)
0.25 + 0.125 (7/7)	different dose each consecutive day	11 (10.58%)
5 × 0.25 + 2 × 0.125 (7/7)	lower dose taken on weekend	3 (2.88%)
0.25 (6/7)	drug-holiday on Sunday	4 (3.85%)
3 × 0.25 + 3 × 0.125 (6/7)		1 (0.96%)
0.125 (6/7)		22 (21.15%)
0.25 (5/7)	drug-holiday on Wednesday and Saturday	1 (0.96%)
2 × 0.25 + 3 × 0.125 (5/7)		1 (0.96%)
0.125 (5/7)		27 (25.96%)
0.125 (4/7 + 3/7)	dose taken every second day	15 (14.42%)

accepted in this case. In the second analysis, 32 pairs of pre- and post-drug holiday (48 h) were tested, and the tested hypothesis was rejected, as $p = 1.91 \cdot 10^{-5}$ with mean difference between trough levels of approximately 0.22 ng/mL (Table 3). The estimated correlation between two time points was $r = 0.887$, $p < 0.001$ suggesting that the test was appropriate.

Data were analyzed by multiple linear regression using stepwise method, where dependent variable was the ratio of given dose (μg/h) and measured trough concentration (ng/mL). Only patients with non-missing values for all variables were included in the analyses. According to the results, 33.3% of variance in the dependent variable was collectively explained by CLcr and IBW, and the overall relationship was significant ($F_{2.83} = 20.74$, $p = 4.94 \cdot 10^{-8}$). The ratio of digoxin dose and concentration was mainly predicted by CLcr, and to lesser extent by patient's IBW (Table 4). Regression coefficients and standard errors of the predictors together with their correlations and collinearity statistics are reported in Table 4.

DISCUSSION

In some health-care systems digoxin is prescribed with interrupted schedule in order to prevent digoxin toxicity. It represents a discrepancy between official guidelines and this rather empirical digoxin dosing. There are few published articles that support presence of interrupted dosing regimens in other health systems as well (14-17). Nevertheless, these dosing regimens raise an important issue from pharmacokinetic/pharmacodynamic perspective, namely reaching and maintaining steady-state concentrations between doses, and whether adequate control of clinical signs and symptoms is possible. From our perspective, the main reason for dosing digoxin with drug holiday is in limited availability of strengths lower than 0.25 mg, and no routine monitoring of concentrations, as other authors agreed as well (14, 15, 17). Based on our results, digoxin weekly dose was in range from 0.375 to 1.75 mg, what is in agreement with other data considering patients' age and renal function. As given in Table 2, the lowest administered digoxin dose is 0.125 mg e.g., 1/2 of tablet. The optimal dose of 0.0625 mg requires 1/4 of 0.25 mg tablet, which is unacceptable from the perspective of the product characteristics. Tablet formulation used for dosing the patients can be split in halves (13), and, as aforementioned, medical professional staff in the hospital was responsible for dividing the tablets. Nonetheless, splitting tablets may produce uneven breaking, breaking into more than 2 parts, powdering and crumbling specially when splitting by hand or using kitchen knife, what is common when patients are home-based. This may lead to inaccurate dosing and consequently clinical implications, especially in drugs with narrow therapeutic index (21, 22). Nevertheless, when only one digoxin dosing strength is registered, physicians individualize dosing regimen by varying dosing interval and/or prescribe half of 0.25 mg tablet. During the study period, different dosing regimens were observed in our clinical settings (Table 2), and efficacy and safety of each of them were determined by monitoring of the drug levels, and clinical signs and symptoms, as well. In 5 patients digoxin toxicity was observed dur-

Table 3. Results of dependent t-test.

Digoxin level (ng/mL)	Mean ± standard deviation	95% Confidence interval of difference	t value	p value
pre-drug holiday	1.28 ± 0.54	0.133 - 0.316	5.013	< 0.01
post-drug holiday	1.05 ± 0.41			

Table 4. Results of multiple regression analysis.

Model	Coefficients (Standard error)	Significance	Part correlations	Tolerance statistics
Constant	4.147 (1.621)	0.012		
Creatinine clearance[a]	0.087 (0.014)	$7.48 \cdot 10^{-9}$	0.577	0.824
Ideal body weight	-0.071 (0.029)	0.016	-0.220	0.824

[a]calculated according to Cockroft-Gault formula which included total or ideal body weight if body mass index was below or above 25 kg/m^2, respectively.

ing the study. When arrived to the hospital, in 2 patients digoxin levels were far above (5.8 and 6.9 ng/mL) the upper range, and the therapy was discontinued. In other patients, drug levels were less than 2 ng/mL, but toxicity was present according to the presence of nausea, vomiting and bradycardia. All of them had normal electrolyte status, normal thyroid function tests, and no other metabolic conditions (hypokalemia, hypercalcemia, hypomagnesemia) that make cardiac tissue sensitive to digoxin. The available literature suggests that under similar circumstances digoxin toxicity may occur.

In this study, we observed variability of 35.35% and 38.75% in achieved digoxin serum concentrations in HF and AF + HF patients, respectively. As highlighted in introduction, several different reports refer to dissimilar acceptable range of digoxin. Based on our results, lower digoxin levels are achieved in patients with HF than HF + AF indication, what is in accordance with the recommendations (11). However, in 40.63% patients with HF indication trough concentration was in range 0.5-1.2 ng/mL, while in remaining patients drug levels were between 1.2 and 2 ng/mL, raising the question if redefined digoxin target levels had any influence on our routine clinical practice. Goldberger et al. discussed the factors that may contribute to the existing failure in adopting new evidence of digoxin dosing into everyday practice (11). With the intention to be sufficiently critical, but at the same time comprehending the limitations in dosing and lack of therapeutic drug monitoring in our health system, we recognized that there is no possibility to assume the overdosing as no signs and symptoms of toxicity were observed. Hence, risk of digoxin regimen due to high concentrations was undetectable in our clinical settings. Results of *post hoc* DIG trial determined that drug concentrations above 1.2 ng/mL in HF patients, increase mortality rate and hospitalizations (8, 10, 23). As these treatment outcomes are long-term defined, and cannot be seen in the study design we performed, the routine practice based solely on clinical conditions may not support administered digoxin dosing regimens. Similarly, for patients with HF + AF indication concentrations > 2 ng/mL showed a good tolerability without side effects. Based on this result, digoxin concentrations > 2 ng/mL seem acceptable as no clinical manifestations of toxicity were observed. In addition, for AF + HF indication, digoxin range is not consistent among available sources, ranging from 0.8 up to 2 ng/mL (11). Hence, till new evidence appears, it may be justifiable to administer regimen based on the dose that will be well tolerated.

We analyzed the difference in digoxin serum trough concentration before and after the drug-holiday, and the result was statistically significant. Post-drug holiday digoxin level was statistically significantly lower than pre-drug holiday. This indicates rather variable trough concentrations in interrupted dosing when steady-state was assumed according to the length of the therapy. Sadray et al. (15) observed a sub-therapeutic concentration after the holiday in 22.77% of patients whose pre-drug holiday concentrations were in the therapeutic range contrary to our results. In accordance with pharmacokinetic principles, dosing with constant dose and dosing interval can establish steady concentrations. Based on our results, stable steady-state is unlikely when digoxin is administered with different dosing intervals between doses. Since digoxin is a narrow therapeutic index drug, in order to maintain the concentrations in the constricted range, it would be more appropriate to dose less more frequently than more drug less frequently. In another words, administration of 0.0625 mg/24 h is preferable over 0.125 mg/48 h. In spite of that, the unavailability of needed dosage units on the market restricts these considerations in the routine practice.

As digoxin shows high pharmacokinetic variability, we studied the influence of patients' demographic and clinical characteristics on the ratio of dose and trough levels. Functional capacity of renal system is physiologically decreased in older patients (≥ 65 years), who were predominant subjects in this study (73.08%). Consequently, as digoxin is renally excreted, our results confirm that renal function, expressed *via* CLcr, significantly influences the ratio of given dose and concentration (Table 4), what is in accordance with earlier publications (24-28). Renal impairment may increase the risk of digoxin toxicity by decreasing digoxin clearance and prolonging its half-life (7). Hence, this suggests that dosage interval should be prolonged or maintenance dose lowered in elderly patients. As expected, lower daily doses were observed in older than adult patients, with mean values 0.113 ± 0.0448 *versus* 0.164 ± 0.071 mg/day, respectively. In addition to kidney function, patient's IBW influenced the ratio of digoxin dose and serum concentration (Table 4). According to Bauman equation, IBW in addition to CLcr improved the overall fit of the model, and proposed dosing nomogram supports our results (24). The principle of IBW-based dosing digoxin in obese patients was proposed by other authors as well (29). Lean body weight is included in some dosing recommendations (5, 25); however, our results highlight the importance of IBW over lean body weight.

If considering typical patient with IBW = 70 kg, our model suggests 0.125 mg/day in achieving 0.7 ng/mL in patient with CLcr = 120 mL/min, while in patient with CLcr = 60 mL/min dose of 0.0625 mg/day or 0.125 mg/48 h would be preferred, and when CLcr decreases to 30 mL/min appropriate dosing would be 0.0625 mg/48 h. These suggestions imply that dosing based on patients' characteristics rather than interrupted regimen could achieve steady digoxin levels. Our results are in compliance, but they give more precise recommendations in relation to patients' characteristics, with labelled recommendations (12, 13), and they support results of other studies (24, 27).

Digoxin is usually add-on treatment rather than first-line drug in treatment of AF and/or HF. Concomitant drugs analysis has shown that all patients in our study were prescribed at least 3 drugs. Additionally, due to other comorbidities, polytherapy is regularly present in these patients. Hence, concomitantly given drugs can interact with digoxin, based on pharmacokinetic and/or pharmacodynamic mechanism. If concomitantly administered drug is known to interact with digoxin, dosing regimens in our settings were optimized based on clinical signs and symptoms. During the analysis, digoxin pharmacokinetic interactions were in focus. According to the results, no effect of concomitantly given drugs (Table 1) was observed; even some of them show a significant effect on digoxin serum levels (5-7). This result may be due to the fact that in the studied population great interindividual variability can be observed, and great number of co-administered drugs was present in the treatment. Consequently, heterogeneous characteristics limit the possibility to assess and quantify the sources of digoxin pharmacokinetic variability using multiple linear regression analysis.

CONCLUSIONS

Interrupted schedule shows greater variability in drug levels comparing to continuous dosing, and it additionally cause difficulties in reaching and maintaining steady trough levels between doses. Interrupted schedule of digoxin may seem acceptable due to the fact that there are no oral forms with lower digoxin strengths on the market. Digoxin dosing has to be carefully guided based on target levels and not solely on clinical signs and symptoms, but taking into account CLcr and IBW. Results from this study may guide further re-evaluation of digoxin posology in the clinical settings in order to ensure therapeutic digoxin level with optimal clinical outcomes.

Conflict of interest statement

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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BLOOD COUNT IN PATIENTS WITH MULTIPLE SCLEROSIS TREATED WITH MITOXANTRONE IN SHORT TIME OBSERVATION

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Abstract: Multiple sclerosis (MS) is an inflammatory, demyelinating disease that affects the central nervous system. Etiology of MS is undiscovered but it is assumed that both genetic and environmental triggers play an important role in disease pathogenesis. Mitoxantrone (MTX) is an antracycline antibiotic that is used in oncologic treatment of breast, prostate, liver, ovarian and stomach cancer. MTX is also effective in treatment of primary and secondary progressive multiple sclerosis and in relapsing – remitting subtype of disease with no reaction for other drugs therapy. In treatment of MS drug is given intravenously in a dose of 12 mg/m^2 in three months intervals to maximal dose of 120-140 mg/m^2 of body surface. MTX treatment can cause transient reduction of leukocyte, erythrocyte and thrombocyte number in blood but the most dangerous side effect of MTX treatment is therapy related acute leukemia (TRAL). The aim of this study was to evaluate influence of MTX treatment on complete blood count in multiple sclerosis patients. Seventy two patients with multiple sclerosis treated with mitoxantrone from 2002 to 2014 took part in this study. Control group comprised 60 patients with multiple sclerosis who weren't given immunomodulatory treatment. In this study, amount of leukocytes, erythrocytes and thrombocytes after MTX treatment was compared to those before treatment and in control group. Six patients were withdrawn from the study because of leucopenia. A decrease of leukocytes, erythrocytes and thrombocytes number after MTX treatment was observed in comparison to control group and value before treatment. The decrease of erythrocytes number after MTX treatment was statistically significant. The most frequent side effect of mitoxantrone treatment is transient, asymptomatic leucopenia. Therapy related acute leukemia and other life-threatening complications weren't observed in the study group.

Keywords: mitoxantrone, multiple sclerosis, leukemia

Multiple sclerosis (MS) is an inflammatory, demyelinating disease that affects the central nervous system (CNS). It is considered to be a frequent cause of neurological disability in young adults (1, 2). In MS there is inflammation that leads to the damage of the myelin sheath surrounding the nerve fibers of the CNS (3, 4). Etiology of MS is undiscovered but it is assumed that both genetic and environmental triggers play an important role in disease pathogenesis (5). The histological hallmarks of the disease are multifocal inflammatory lesions in brain white matter. Autoreactive T and B cells are involved in that process connected with microglial reactivity and leukocyte infiltration. All these responses are tightly regulated by cytokines, chemokines, adhesion molecules. They can be also modified by immunomdulatory drugs like mitoxantrone (MTX) (6, 7). Mitoxantrone is effective in primary, secondary progressive and relapsing remitting MS when other therapies remains ineffective (8, 9). MTX is also used in oncologic treatment of breast, prostate, liver, ovarian and stomach cancer. Since a few years it is successfully applied to patients with primary and secondary multiple sclerosis who didn't respond to another drug treatment (9, 10). MTX inhibits topoisomerase II, DNA and RNA synthesis and cells proliferation (9, 10). It reduces lymphocyte T and B and antibodies synthesis that is observed even one year after the end of treatment (9, 11). The most frequent side effects of MTX therapy are: nausea and vomiting, hair loss, increased risk of respiratory and urinary tracts infections, menorrhagia disorders, leucopenia, thrombocytopenia, anemia, liver enzymes and bilirubin level elevation. The most dangerous side effects of MTX treatment are: cardiotoxicity and therapy related acute leukemia (TRAL) with the incidence of 1 per 300 treated patients (10, 12). The aim of the study

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was to evaluate the influence of MTX treatment on total blood count in MS patients.

METHOD

Seventy two (72) people with MS treated in Neurology Department in Military Institute of Medicine from 2002 to 2014 took part in this study. They were in age ranged from 25 to 63 years old, approximately 47 ± 10.77 years (women 47.9, men 44.6 years). In investigated group, there were 52 women (72%) and 20 men (28%). Among participating people, 56% suffered from primary progressive MS, 7% had secondary progressive MS and 37% had relapsing – remitting subtype of disease. The mean time of symptoms duration was 12 years ± 7.34. In control group, there were 60 people in age ranged from 19 to 65 years old, who weren't given immunomodulatory treatment. The mean age in investigated group was 36.3 ± 6.8 years (women 37.6, men 32.7). In control group there were 43 women (71.7%) and 17 men (28.3%). In control group primary progressive MS had 7 patients (11.6%), secondary progressive had 7 people (11.6%) and in case of 46 relapsing – remitting subtype of disease was diagnosed (76.6%) (Table 1). The mean duration of symptom was 6.8 ± 6.53 years (from 1 to 28 years). MTX was given intravenously in dose 12 mg/m^2 of body surface area to maximal dose of 120-130 mg/m^2 in three months intervals. Before each MTX dose total blood count was done. The amount of red (RBC) and white blood cells (WBC) and thrombocytes (PLT) was evaluated. These results were compared with those in control group and before MTX treatment.

RESULTS

The mean value of WBC before MTX treatment was 7.07 (± 2.38) × 10^3. The mean value of WBC after end of MTX treatment was 6.68 (± 2.34) × 10^3. The mean WBC value in control group was 7.26 (± 3.05) × 10^3 at the beginning and 7.37 (± 4.4) × 10^3 at the end of observation. There was no statistically significant decrease of WBC at the end of MTX treatment in comparison to value before MTX therapy ($p = 0.709$) and in control group ($p = 0.509$) (Table 2).

There was no statistically significant correlation between total dose of MTX and number of WBC in MS patients' blood in e-Pearson test ($p = 0.736$). Six patients were excluded from the study because of leucopenia.

The mean value of RBC before treatment was 4.68 (± 0.61) × 10^6 while at the end of treatment was 4.5 (± 0.38) × 10^6 (Table 3).

Statistically significant decrease of RBC number in MS patients blood compared to number of

Table 1. Multiple sclerosis subtypes of disease in group treated with MTX and in control group.

SM course	Number of patients treated with MTX	Number of patients with no MTX treatment	% of total patients treated with MTX	% of total patients with no MTX treatment
Primary progresive	40	7	55.56%	11.66%
Relapsing-remiting	27	46	37.50%	76.66%
Secondary progressive	5	7	6.94%	11.66%

Table 2. Mitoxantrone treatment influence on white blood cells amount in multiple sclerosis patients' serum in comparison to control group - analysis of variance $F(1. 82) = 0.14$; $p = 0.709$, $F(1. 82) = 0.45$; $p = 0.509$.

	MTX treatment	Average × 10^3	Standard deviation
INITIAL VALUE	YES	7.07	2.45
	NO	7.26	1.91
	TOTAL	7.11	2.34
FINAL VALUE	YES	6.68	2.37
	NO	7.37	4.40
	TOTAL	6.82	2.88

Table 3. Red blood cells changes in multiple sclerosis patients' serum before and after MTX treatment (analysis of variance F(1. 22) = 5.09; p = 0.034).

	Average $\times 10^6$	Standard deviation	Min $\times 10^6$	Max $\times 10^6$
Before MTX treatment	4.68	0.61	3.69	6.57
After MTX treatment	4.50	0.38	3.77	5.22

Table 4. Influence of MTX treatment on thrombocytes amount in multiple sclerosis patients' blood in comparison to control group (analysis of variance F(1. 80) = 0.41; p = 0.524, F(1. 80) = 2.59; p = 0.112).

	MTX therapy	AVERAGE $\times 10^3$	Standard deviation
INITIAL	YES	244.54	68.84
	NO	232.55	58.77
	TOTAL	243.07	69.43
FINAL	YES	236.48	68.42
	NO	253.93	65.28
	TOTAL	239.38	68.03

RBC before treatment was observed (p = 0.034). Statistically significant correlation between total MTX dose used in treatment and RBC number in blood of MS patients was not observed. Anemia demanding exclusion from the study was not observed.

The mean PLT number before treatment was 245 (± 69) × 10^3 and at the end of MTX treatment was 236 (± 68) × 10^3. The mean PLT number at the beginning of observation was 232.55 × 10^3 (± 58.77) and was 253.93 ± (65.28) × 10^3 at the end of the trial. Statistically significant changes in PLT number before treatment compared to the value at the end of therapy was not observed (p = 0.618). Statistically significant deterioration in PLT number after MTX treatment in comparison to PLT number before treatment also was not observed (p = 0.127) (Table 4).

Statistically significant correlation between total MTX dose given to patients and PLT number in blood was not observed in e-Pearson test.

DISCUSSION

MTX is a drug that has antinflammatory and immunomodulatory properties. DNA molecules can be damaged by MTX, what can lead to acute myeloid leukemia (AML). MTX toxicity is a result of DNA recovery mechanisms activity inhibition (13). TRAL is a rare and dangerous complication of MTX treatment with incidence of 1/300 patients. First case of TRAL was described in 1998 year (10). That risk is even higher in case of oncologic patients (14). Its peak of incidence is from 3 month to 5 years after end of MTX treatment (10, 15). Time from the beginning of MTX therapy to TRAL diagnosis is approximately 3 years. In USA from 2003 to 2007 year, 39 cases of TRAL were diagnosed (7 people died) (14). In another study correlation between TRAL incidence a total MTX dose given to patients, age, sex, time of MS symptoms and concomitant drugs was not observed (15). Higher risk of TRAL maintained for five years after the end of MTX treatment. Exact incidence of that disease remains unknown because a majority of trials last shorter than five years. Sadliq described case of chronic leukemia related to MTX treatment (16). In present study, any case of TRAL was observed during MTX treatment. Further study needs to be done to evaluate risk of TRAL in five years after the end of MTX treatment. A decrease of WBC, RBC and PLT number is more frequently observed. Wundes observed in his study statistically significant deterioration of WBC and PLT number two weeks after MTX infusion in comparison to values before treatment. In case of majority of patients, number of WBC, RBC, PLT got back to normal before next MTX infusion. In the group of 96 patients four were excluded from the study because of leucopenia and one because of thrombocytopenia. Severe anemia was not observed in that study (17). Impact of total MTX dose on the risk of hematologic complications remains unknown. Ellis and Boglid proved that this risk is higher when MTX dose administered is higher than

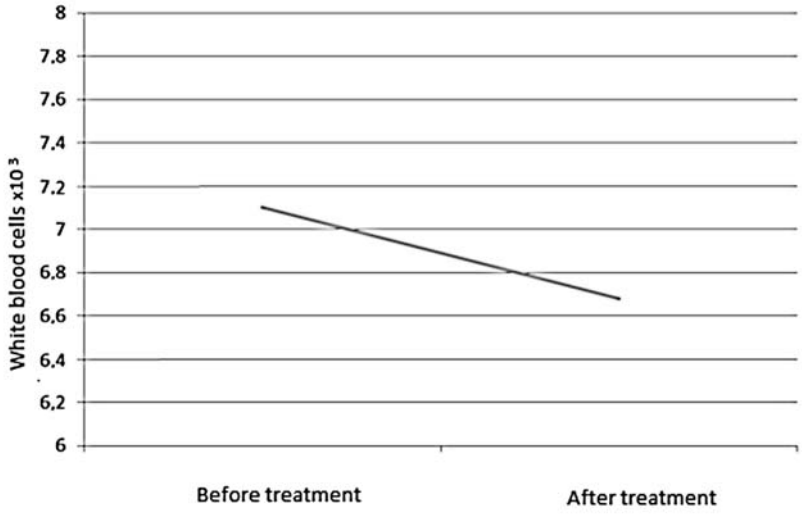

Figure 1. White blood cells amount after MTX treatment in comparison to amount before treatment

60 mg/kg (18). In Hartung's study, leucopenia was observed in case of 19% patients treated with MTX in dose of 12 mg/m^2 of body surface (19). In presented group, a decrease of white blood cells number in comparison to number before treatment was observed but it was not statistically significant. Six patients were excluded from the study because of leucopenia (in all cases number of WBC returned to normal after the end of treatment) (Fig. 1).

There is a lack of data analyzing the frequency of anemia and thrombocytopenia in MS patients treated with MTX. In presented study anemia and thrombocytopenia demanding exclusion from the study were not observed. Thrombocyte number after treatment was lower compared to value before MTX treatment and to that in control group but it was not statistically significant. Correlation between MTX dose and thrombocyte number was not observed.

Statistically significant decrease of erythrocytes number after MTX treatment compared to number before therapy was seen in present study. Number of RBC didn't correlate with MTX dose administered to the patient. Anemia demanding exclusion from the study was not observed.

CONCLUSIONS

This study revealed that MTX should be considered as a safe and effective treatment in patients with severe course of MS. The most important side effect of MTX therapy, connected with its mechanism of action is transient leucopenia. The risk of getting TRAL remains low. Further studies need to be done to evaluate long-term impact of MTX on hematopoietic system.

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GENERAL

PHARMACEUTICAL CARE FORM APPLICATION IN ELDERLY PATIENTS RESEARCH

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Abstract: Many patients, especially elderly, very often don't inform family physicians about drugs prescribed by other specialist and use a number of preparations to self-medicate. These actions may supply negative consequences in recovery process and can be significantly reduced through the implementation of a well-run pharmaceutical care (PC) in the community pharmacy. Therefore, it is necessary to design proper PC documentation, which allows for comprehensive gathering information needed to carry out the analysis of relevant factors impacting on the prescribed pharmacotherapy effectiveness. The aim of this study was to develop the proper documentation to conduct PC in community pharmacy and verification of its correctness in the group of elderly patients, who is receiving treatment from family physician and additionally at least two specialized doctors. Also an assessment of PC implementation validity as a specialized service was presented. A study was carried out from January 2014 to June 2015. The research covered 54 elderly (41.0% men and 59.0% women) in Jarocin (Poland). Pharmacist was filling in the designed PC form, which subsequently was analyzed mainly for pharmacotherapy problems. The study indicated that 48.1% of patients were counselled by at least 3 specialists; 66.7% of patients were treated by cardiologist and 55.6% by the ophthalmologist; 75.9% of participants had hypertension. 42.6% of the patients used 10 or more pharmaceuticals and 24.1% took 8 or 9 drugs. Moreover, 20.4% of participants used drugs irregularly and 7.4% of them took too low doses of medications. Additionally, during the pharmacotherapy analysis, very significant and significant drug-drug interactions were noticed (1.1% and 18.4%, respectively). Very significant drug-food interactions occurred in 1.4% cases and significant in 3.3% cases. Total number of detected interactions were 553. The substantial analysis of participants' data allowed for 48 patients selection for pharmaceutical counselling. Six patients were involved into the group where PC process was reasonable. These patients often used 10 or more drugs ($p = 0.0026$), discontinued using medication without doctor consulting ($p = 0.0201$) and did not follow up the prescribed pharmacotherapy ($p = 0.0351$). This study provides new data about implementation of PC in Poland. The designed PC documentation may be helpful for proper analysis of the patient's pharmacotherapy to avoid drug problems and to improve elderly patients' quality of life. This project may contribute to PC implementation in Polish community pharmacy.

Keywords: pharmaceutical care, elderly, documentation, drug problems, non-adherence

Elderly patients often receive care from several doctors which makes the scheme of pharmacotherapy more complicated. This is overlapping with the effect of over the counter medications and dietary supplements self-treatment, which additionally increases the risk of drugs problems (1). For this reason, for many years, pharmaceutical care (PC) is practiced all over the world as a documented service, where pharmacist collaborates with the patient and the physician and, if necessary, with other health care professionals and ensures the proper conduct of pharmacotherapy in order to achieve specific outcomes, which improve the patient's quality of life (2). It is especially important for elderly patients, who very often suffer from chronic disorders and multi-disease. It requires using large

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amount of drugs and leads to polypharmacy (3). Proper patient adherence to the doctor's recommendation is meaningful for therapy effectiveness. Non-adherence contributes to the failure of medical treatment. This problem affects many patients e.g., almost 25.0% of them reduce alone the number of drugs used without doctor's consultation. These actions may lead to serious consequences such as pharmacotherapy failure, prolonged treatment or exacerbate patient's health condition, hospitalization and in the most severe cases death (4).

PC is an integral element of patient's health care which requires proper documentation for its conducting. It has a crucial impact on the running of the PC service, because it allows to collect patient's health and lifestyle data in a proper way. Then, it gives the possibility of the detailed drug analysis arrangement which improves patients' pharmacotherapy (5-7).

The aim of this study was to prepare a standard draft form to conduct PC in community pharmacy and verification of its correctness in the group of elderly patients who is treated by family physician and at least two specialized doctors. The essence of this study was to determine the PC implementation validity as a specialized service carried out in community pharmacy among elderly patients.

MATERIAL AND METHOD

The research was conducted in Jarocin (Poland) between January 2014 and June 2015. This

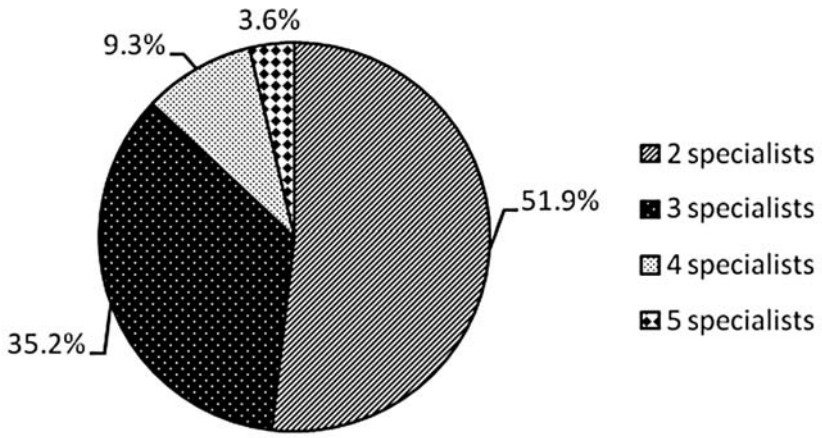

Figure 1. The number of physicians who counselled the patients, n = 54

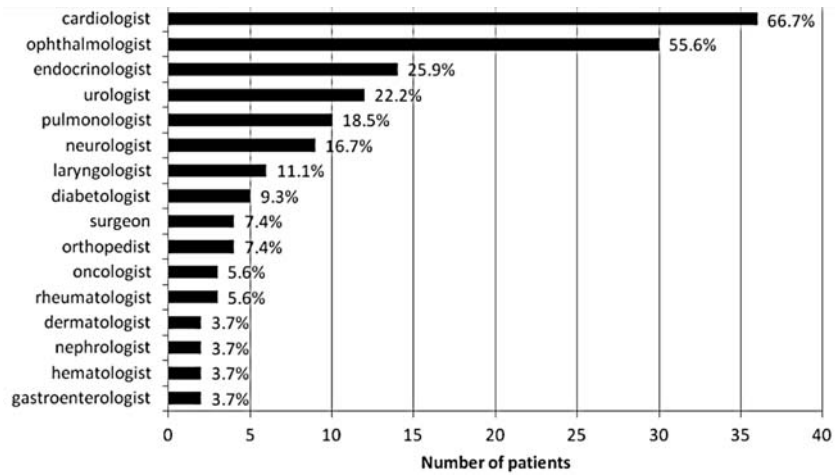

Figure 2. The specializations of physicians involved in patients' treatment, n = 54

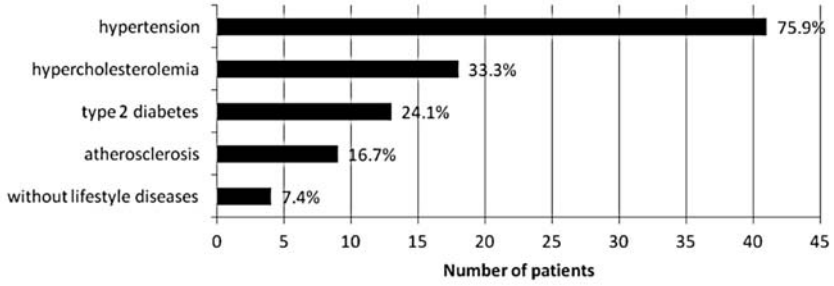

Figure 3. Valuation of selected patients' lifestyle diseases, n = 54

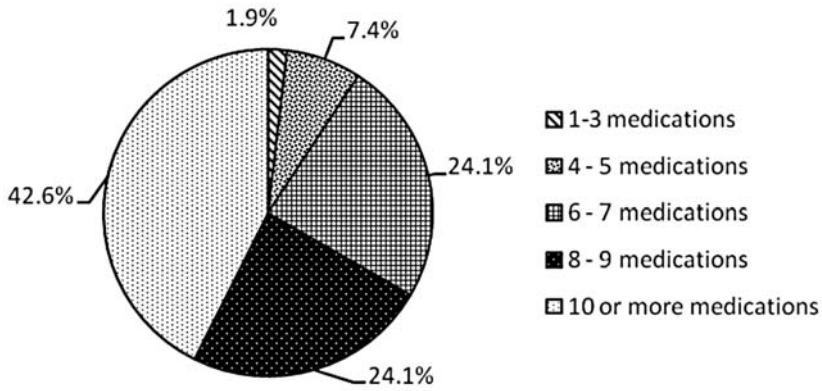

Figure 4. The number of medications used by patients, n = 54

study was based on the PC author's draft form and was filled in by the investigator. There were involved 54 elderly patients (59.0% women, 41.0% men) treated additionally by two or more specialized doctor. Elderly patients were referred from family doctors working in Primary Care Physicians Clinic. Patients aged 65 year and more were mostly in the 65-74 years and 75-84 years subgroups (48.2% and 44.4%, respectively). The rest of the patients had more than 85 years. About 60.0% of the study group was characterized by a basic or vocational education. The rest declared secondary or higher education.

The study was based on the designed form to conduct PC. During the interview patient's basic information was evaluated. The collected data were analyzed among others in terms of the proper pharmacotherapy and interaction occurring between the prescribed drugs and food components. Substantive analysis where very significant and significant interactions were detected allowed for patients selection where PC implementation is reasonable. Pharmaceutical counselling was provided for each patient who wasn't classified to PC. The study received Ethical Board revision and acceptance.

The results were statistically analyzed with the use of Statistica 10.0 application (StatSoft®). To compare the pharmacotherapy risk factors in the two groups of patients with and without indication for PC test for proportions was applied. All statistical analyses were performed at $p < 0.05$.

RESULTS

The study indicated that 48.1% of patients were counselled by at least 3 specialists (Fig. 1); 66.7% of patients were treated by cardiologist, 55.6% by the ophthalmologist and 25.9% by the endocrinologist (Fig. 2). 75.9% of patients had hypertension and 33.3% had hypercholesterolemia (Fig. 3). 42.6% of the patients used 10 or more pharmaceuticals and 24.1% took 8 or 9 drugs (Fig. 4). Moreover, 20.4% of participants used drugs irregularly and 7.4% of them took too low dose of medications (Fig. 5). Additionally, during the pharmacotherapy analysis, very significant and significant drug-drug interactions were noticed (1.1% and 18.4%, respectively). Very significant drug-food interactions occurred in 1.4% cases and significant in 3.3% cases. Total number of detected interactions were 553 (Fig. 6).

The substantial analysis of participants' data allowed for 48 patients selection for pharmaceutical counselling, 6 patients were involved into the group where PC process was reasonable (Table 1). These patients often used 10 or more drugs (p = 0.0026), discontinued using medication without doctor consulting (p = 0.0201) and did not follow up the prescribed pharmacotherapy (p = 0.0351).

DISCUSSION AND CONCLUSION

The elderly patients, as it was proved in this study, compose a group with many pharmacotherapy problems. Very often it is due to many doctors consultations and patients' non-compliance (1, 8). In this study, 76.0% of patients had hypertension and about 30.0% had hypercholesterolemia. 48.1% of patients were counselled by at least 3 specialists and 66.7% of patients were treated by cardiologist and 55.6% by ophthalmologist. Comparable results in elderly population were presented in PolSenior study (9). Nowadays, patients are counselled by many specialists and very often they don't inform physicians about other consultations. It leads to many problems especially concerning pharmacotherapy. In such cases, pharmaceutical advice, which is commonly practiced in Polish community pharmacy, does not approve sufficient effect and so becomes a necessity to implement a well-run PC as it is applied in many countries (10-12).

This research presented that approximately 2/3 of the patients used 8 or more pharmaceuticals. Additionally 20.3% of participants used drugs irregularly and 7.7% of them took too low dose of medications. According to the study from France, overuse (77.0% of the patients), underuse and at minor degree of misuse were mostly evidenced at elderly patient group (13). O'Connell et al. demonstrated that about 23.0% of patients took wrong doses of medications (14). Current investigation proved additionally that polypharmacy is a popular problem at elderly and this is consistent with many studies (9,

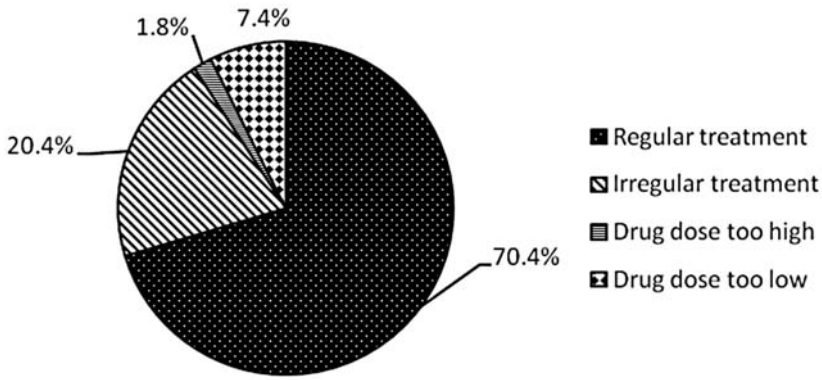

Figure 5. Patients' adherence to recommended treatment, n = 54

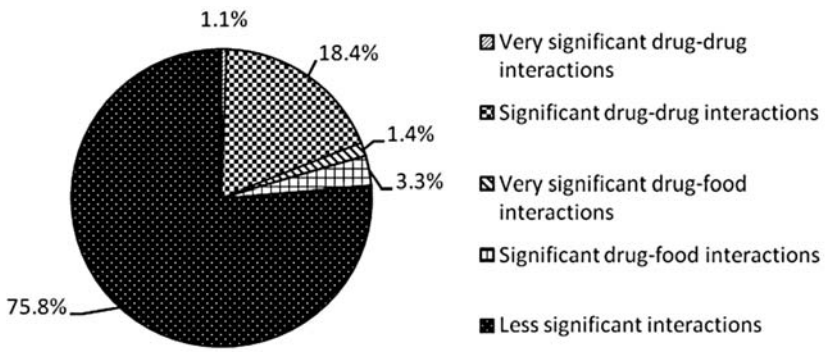

Figure 6. Division of detected interactions among the patients, n = 553

Table 1. The distinction of patients who required PC according to the risk factors.

Risk factors	n = 6		n = 48		p-Value
	Patients who required PC		Patients who didn't require PC		
	n	n (%)	n	n (%)	
Application of 10 or more medicines	6	100.0	17	35.4	0.0026*
Self discontinuation of treatment	3	50.0	6	12.5	0.0201*
Not following up the prescribed pharmacotherapy	4	66.7	12	25.0	0.0351*

*$p < 0.05$

15-17). Moreover, this issue is highly manifested in the elderly women population (18).

Unnecessary drugs increased the risk of non-adherence and interactions occurrence (19). Some studies indicated that at least 20.0% of elderly had low adherence to treatment (20-22) so it resulted e.g., in significant drug-drug interactions (46.0% cases) and in potential herb-drug interactions (33.0% cases) (23). In study from 2016, 70.0% of participants used at least one potentially inappropriate medication (24). Additionally, Ahmad et al. followed drug related problems from hospital discharges. There were 992 potential problems with medication which were observed in 340 patients. Ten percent of these cases were drug-drug interactions (25).

Another significant problem found in this study was an irregular treatment. The study of Roberts et al. confirmed that it could be connected even with 70.0% of patients (26). Additionally, the most common mistakes made by patients at home were: incorrect dosage, forgetting, mixing up medications, failing to recall indications and taking out-of-date or inappropriately stored drugs (27). So, it is very important to provide reasonable PC in community pharmacy with drug history documentation which include also over the counter medications and health supplements analysis. Then, pharmacist can prepare drug use review to search drug-drug and drug-food interactions and suggests medicine with lower risk of interactions to optimize drug therapy (23).

Essential element of the study were preparation of adequate PC documentation and proper patients' indication for PC or pharmaceutical counselling. The selected patients for PC often used 10 or more drugs, discontinued using medication without doctor consulting and did not follow up the prescribed pharmacotherapy. The PC recommendation and documentation, in general, are similar in many countries**,** but still there are different types of medication review procedures (28-30). Thus, there should be organized a trial to get a better cooperation between countries to obtain standardized medication review practice nationally and internationally developed (28).

The obtained data confirm that there is a need of PC implementation especially for elderly. This documented analysis should be an integral part of patient's health care because physician refers patient or due to patient's request. Finally, it should be refunded by the National Health Fund. Implementation of PC brings many benefits for the patients pharmacotherapy mainly for improving the quality of elderly lives.

Acknowledgment

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ROLE OF THE INTERNATIONAL ORGANIZATIONS IN PREVENTING THE COUNTERFEIT MEDICINES ENTRY INTO THE WORLD MARKETS

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Abstract: 30 years have passed since Conference of Experts on the Rational Use of Drugs was held in Nairobi, Kenya, from 25 to 29 November 1985, where the problem of counterfeit medicines was mentioned as the international for the first time. The problem of counterfeit medicines is not only a major threat to public health and national and private economy, but also it is of great interest for key decision-making actors at the international level. The authors analyzed what has been done since that time by international organizations. Combating the counterfeiting of medicines cannot be successfully achieved by the health sector alone – World Health Organization (WHO), – so the efforts of the other United Nations (UN) organizations relevant to counterfeiting were in need and were studied in the article: World Intellectual Property Organization (WIPO), World Trade Organization (WTO), World Customs Organization (WCO), United Nations Office on Drugs and Crime (UNODC), etc. Today WHO is unable to coordinate all their activities, so the few existing proposals for establishing a new mechanism of international cooperation have been examined. Will the MEDICRIME Convention that will enter into force on January 1, 2016 be the start of the new era in the combating with the counterfeit medicines? - the authors offered their vision on the international developments.

Keywords: counterfeit medicines, falsified medicines, international organizations, international cooperation, public health, the MEDICRIME Convention

Abbreviations: ACTA - Anti-Counterfeiting Trade Agreement, CCP - Container Control Programme, CoE – Council of Europe, EDQM - European Directorate for the Quality of Medicines and HealthCare, EU – European Union, GHD - Global Health Diplomacy, IMPACT - International Medical Products Anti-Counterfeiting Taskforce, Interpol - International Criminal Police Organization, IP - intellectual property, MEDICRIME convention - Council of Europe Convention on the counterfeiting of medical products and similar crimes involving threats to public health, MSM - member state mechanism, TRIPS - Agreement on Trade-Related Aspects of Intellectual Property Rights, UN - United Nations, UNODC - United Nations Office on Drugs and Crime, UNTOC - United Nations Convention against Transnational Organized Crime, WCO - World Customs Organization, WHO - World Health Organization, WIPO - World Intellectual Property Organization, WTO -World Trade Organization

The counterfeit medicines are multi-faceted and complicated problem. Different interests of developed and developing countries, multinational state actors and international organizations, civil society and business are actively involved in different aspects of the issue (1).

One of the problems is that there are not any special organization and finance for making the systematic reports about it, so one can have only general understanding of the problem's extent. However, there are some points for making a general idea about its geographical expansion and range.

Recent data indicate that counterfeit medicines are mainly produced in China and India. The way by which counterfeit medicines reach patients is not easy: for example, in February 2012, counterfeited Avastin was detected in 19 U.S. medical practices that had traveled through Turkey, Egypt, Switzerland, and the United Kingdom, before reaching the United States, so even in such heavily regulated states as the United States and Europe, counterfeit medicines are now available - primarily through online distributors (2).

The counterfeit medicine market has become broader: it is not only expensive lifestyle medicines but the whole scale of all types of medicines is available on the illegal market (3). Traditionally, regions with weak regulatory structures, such as Africa,

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Latin America and parts of Asia, have been both producers and consumers of counterfeit drugs. Then, falsified medicines are sold all over the world (4). The authors came to the conclusion that in more poor country with the weak regulatory authorities, there is a greater range of counterfeit medicines. No medicine is safe from counterfeiting.

The next problem is that the different terminologies and notions are used for counterfeit medicines. WHO uses the term "substandard/spurious/falsely-labelled/falsified/counterfeit" medical products and "counterfeit" medicines; the European Commission uses the term "falsified" medicines and explain that the term "falsified" is used to distinguish the issue from intellectual property (IP) violations, so-called "counterfeits", UNODC uses the term "fraudulent" medicines – could not agree that "without appropriate and agreed-upon definitions, it is difficult to develop harmonized solutions to address "counterfeits" (5). Under WTO definitions, counterfeits only refer to trademark rather than patent violations and do not take into account quality considerations (6), but it is important for pharmaceutical sphere.

Summarizing up, there are 4 main reasons why there is no strong and broad international cooperation in combating with counterfeit medicines. They are:

- lack of shared goals among stakeholders;
- lack of reliable information and its analysis;
- absence of a clear, internationally agreed terminology and as a result/cause the conflation the problem of medicines quality with intellectual property (IP) rights defense (5);
- poor standards of national regulatory affairs.

Several key international organizations, including WHO, UNODC, Interpol and WCO, have attempted to address the global counterfeit medicines issue.

International organizations relevant to the problem

WHO

In 1948, WHO was established and one of the first questions for deciding was the quality of drugs in international commerce. Nairobi conference (1985) was organized by WHO; next important step was the preparation by WHO in 1996 a Project on Counterfeit Drugs. In 1999 WHO has developed Guidelines for the Development of Measures to Combat Counterfeit Drugs.

WHO has initiated and participated in various activities to raise awareness and contribute to the solution of the issue. WHO provides direct country and regional support for strengthening medicines regulation.

The WHO's historical focus has been on tackling – or sometimes eliminating – specific diseases, but today's view of health is more holistic (7). As the years go by, the WHO's role has challenged as a directing and coordinating authority.

Some of the most common criticisms that are raised in the connection with WHO were:

- Over-politicized to the detriment of its technical functions;
- Too bureaucratic;
- Timid;
- Overstretched/underfunded;
- Conflicted and
- Failing to adapt to change.

The ongoing reforms are aimed at solving these problems (8).

So, one of the reasons for the problem of the counterfeit medicines spread was de facto absence of coordinating center to combat it. WHO has to identify its proper – coordinating – role in the governance of global health. The WHO activities are connected with other international organizations, that are engaged in the problem solving.

WIPO and WTO

On a regular basis WIPO started to participate in actions concerning counterfeit medicines from 2004. WIPO had taken part in some conferences concerning counterfeit medicines but its activities were intensified with WCO, WTO and Interpol in 2004 and it was connected with the First (2004) and Second (2005) Global Congresses on Combating Counterfeiting and Piracy, on the one hand, and with WHO from 2006, on the other hand.

The theme of combating counterfeit medicines was the reason for WIPO's tighter cooperation with WHO after the conference, organized by WHO and held in Rome from February 16 to 18, 2006, which aimed at building stronger and more effective international cooperation in this field. Under the final declaration adopted by the participants, the establishment of an International Medical Products Anti-Counterfeiting Taskforce (IMPACT) was recommended to be organized under the auspices of the WHO. WIPO offered its continued assistance in IMPACT activities to explore further mechanisms for strengthening international action against counterfeit medicines. This includes legislation, regulation, enforcement, technology and risk communication activities (9).

WCO

WCO is an independent intergovernmental organization established in 1952 to enhance cus-

toms' effectiveness and efficiency. WCO represents 179 global customs administrations (10). WCO has recently become a key partner for trade inspections in trafficking of counterfeit medicines.

WCO efforts against counterfeit medicines include also the global Container Control Programme (CCP). Established in 2006, CCP is a joint WCO-UNODC initiative to monitor the movement of cargo shipped by sea.

Interpol

Interpol started to take active part from 2004 – on the Global Congresses that were passed 2004–2013 and it summed up the results of its activities on the global conference where the participants reviewed the progress made over the 10 years (operations) and recommend ways forward for strengthening international collaboration in combating pharmaceutical crime. The necessity of strengthening of the cooperation among law enforcement, health agencies, customs and the private sector was discussed (11).

UNODC

UNODC assists UN Member States in their fight against illicit drugs, transnational organized crime, corruption and terrorism, counterfeit goods and fraudulent medicines, etc. It is the guardian of most of the related global conventions, including 3 drug control conventions, the UN Convention against Corruption, and the UN Convention against Transnational Organized Crime (UNTOC).

Through an extensive network of field offices and its Headquarters in Vienna, UNODC operates in all regions of the world. The three pillars of the UNODC work program are:

- Field-based technical cooperation projects
- Research and analytical work
- Normative work to assist states in the ratification and implementation of the relevant international treaties, etc.

Recognizing the multiple causes of crime, UNODC promotes strategies, plans and programs, which are multi-sectoral, multi-disciplinary, and on which favor civil society participation for crime prevention.

The European Union (EU) is one of UNODC's strongest partners (12).

Resolution 20/6 adopted at the 20th session of the Commission on Crime Prevention and Criminal Justice in April 2011 provides the mandate for UNODC to work on fraudulent medicines. The UNODC had prepared Draft Model Legislative provisions on fraudulent medical products 17. 11. 2014 (13), that was severely criticized by the group of researches headed by Prof. Attaran because this document was written on very low juridical level without normal law-making procedure and it conflates the safety and quality of medicines with an IP enforcement agenda. That has led to significant controversy in the past, particularly for the WHO resolution to set up a member state mechanism (MSM) to tackle the trade in counterfeit medicines in 2012. MSM has been forced to fundamentally rework in the face of criticism from first of all India and Brazil – which alleged it was pandering to an agenda set by the pharmaceutical industry (14).

So, the authors think that the problem is not only in WHO itself, but in the entire structure of the UN – the organization no longer meets the demands of the time. There were enough time for international organizations to use its possibilities to address the issue, but should be noticed that the main activities were started since 2006 – it means after 20 years from the Conference of Experts on the Rational Use of Drugs (1985), where there the issue of counterfeit medicines was announced as international.

Main international treaties relevant to the problem

There are many legal instruments dealing the counterfeit medicines. Complicating matter is that there is no real global consensus on many aspects of the problem IP protection, what "counterfeit" means, etc. and laws to combat such fraud are a patchwork at best (4).

The most discussed international treaties concerning with the counterfeiting are Anti-Counterfeiting Trade Agreement (ACTA), UNTOC and the Council of Europe (CoE) Convention on the counterfeiting of medical products and similar crimes involving threats to public health (MEDICRIME Convention).

ACTA – the multilateral treaty which was secretly negotiated by states without any international organization's participation. It was not adopted because of public protests. This situation reflected the developed nation's point of view on IP issues (15).

UNTOC

UNTOC adopted by General Assembly resolution 55/25 of 15 November 2000, entered into force on September 29, 2003, is the main international instrument in the fight against transnational organized crime (16).

One of the most harmful forms of counterfeit goods is counterfeit medicines. The Convention

included mutual legal assistance for gathering international evidence and extraditing defendants.

UNTOC forms the legal basis for international cooperation against serious crimes and so far counts 182 Member States as parties, approaching universal ratification (17).

The MEDICRIME Convention and the role of the European Directorate for the Quality of Medicines and HealthCare (EDQM)

The CoE is the continent's leading human rights organization. It includes 47 member states, 28 of which are members of the EU. The European Committee was adopted the MEDICRIME Convention in December 2010. There must be 5 states ratifications, including three CoE member states, for entering it into force. The counterfeit medicines represent a global threat, so the Convention is open to non-members of the CoE as well (it is signed by Morocco, Israel and Guinea).

By 13. 10. 2015, it was adopted by 24 states, Croatia is the latest state to have signed the Convention (03. 09. 2015). The MEDICRIME Convention was ratified by 4 member states of the CoE: Ukraine (20. 08. 2012), Spain (05. 08. 2013), Moldova (14. 08. 2014) and Hungary (09. 01. 2014). The last ratification necessary for entering it into force was on May 30, 2015 – members of the National Assembly of Guinea voted unanimously for the ratification of the Convention of the CoE, that they had signed in October 2012. The parliament of Guinea concluded the internal process to ratify the MEDICRIME Convention on September 24, 2015 (18).

The reason of interest and signing is clear: in Guinea, 70% of medicines in circulation are outside the official import system and thousands of people each year become victims of fake antimalarials (19). The 5 required signatures for the entry into force of the MEDICRIME Convention are now achieved and giving it in the future a particular importance.

According to the Article 28, the MEDICRIME Convention shall enter into force on the first day of the month following the expiration of a period of three months after the date on which five signatories, including at least three member states of the CoE, have expressed their consent to be bound by the Convention in accordance with the provisions of the preceding paragraph (20). So, Convention will entry into force on January 1, 2016. Also soon the Convention is expected to be ratified by France, Russia and Belgium.

Convention begins with a list of definitions that allows gaps to be filled in national legislation in cases where criminal and administrative liability for the manufacturing, distributing and selling of counterfeit medicines is not covered by current trademark and patent legislation ('falsified medicines'). The term "counterfeit" under Convention "shall mean a false representation as regards identity and/or source".

The Convention is clearly defined, it does not apply

– against (legal) generics (Article 3),

– against breaches of quality norms, good practices and standards in the manufacture and distribution of medical products that are committed without criminal intent are, nor

– against medical products that were produced or distributed under circumstances that are legal in domestic law, e.g., legal Internet pharmacies (Article 4).

The MEDICRIME Convention aims to protect public health by introducing criminal penalties for certain acts including assisted or attempted counterfeiting, but also by contemplating certain preventive measures and victim protections. It also creates a framework for international cooperation and measures aimed at coordinating efforts at national levels. The EDQM is part of the CoE and aims to lead in protecting public health by enabling the development, supporting the implementation and monitoring the application of quality standards for safe medicines and their safe use (21).

Part of EDQM's strategy is to provide international support for the implementation of the MEDICRIME Convention. So, as soon as it entered into force, the role of EDQM will increase. Signatory states may make use of the expertise and working results co-ordinated by the CoE and its EDQM to support the follow-up of the Convention after its entry into force (18).

So, 30 years of work on the issue could be divided in three stages:

1985–2005 years – the stage of discussions under the auspices of the WHO, the detection range of issues related to counterfeiting (the difference in terminology, aspects relating to intellectual property);

2006–2015 years – the stage of cooperation between the various UN specialized agencies (WIPO, WTO, WCO, etc.), attempts of the single coordinating body establishment (IMPACT, MSM) and the international treaty adopting a series of operations conducted to detect counterfeit medicines.

2016 – entering into force of the MEDICRIME Convention, non-state actors activating and taking them into account by international organizations.

DISCUSSION

There is the variety of policy proposals attempting to address the dangerous counterfeit medicine trade. Each would have its own strengths, weaknesses and associated costs if pursued diplomatically.

Mackey T.K. proposal

The Mackey's proposal was structural one. Mackey and colleagues had noticed the good combination of international organizations with necessary "programmatic activities", where: UNODC combats transnational crime, Interpol performs law enforcement purposes, WCO – customs and border control, and WHO is needed for public health science and analysis (22). Proposal of Mackey had changed over time, in 2012 – UNODC- WHO-Interpol trilateral mechanism is the best way to address this problem (4), in 2013 the same UNODC-WHO-Interpol trilateral mechanism (4, 23), but one more publication of the 2013 has the forth organization – WCO. At the time of writing the article the role of UNODC was high, but in 2014 the situation has changed and the UNODC has had no confidence from developing countries. Mackey positively assessed UNODC support under Resolution 20/6, applicability of UNTOC, and other well-accepted, ratified treaties uniquely situates it to immediately engage and actively promote Global Health Diplomacy (GHD) on the counterfeit medicines issue (22). Mackey underlined that "UNODC is uniquely poised to lead the fight against counterfeit medicines given its status as a specialized agency of the UN with existing normative powers and a mandate to fight counterfeits". He thought that these advantages could enable it to avoid contentious disagreement between member states by focusing on the criminality of the trade, but it did not occur.

The authors agree that WCO has limitations similar to Interpol in that its operations are generally isolated to customs and trade and it does not have normative functions or formalized governance structures. WTO and WIPO would be responsible for hearing trade and IP rights disputes (4).

Mackey stressed that current adoption of the MEDICRIME Convention appeared to be European focused (22), there must be more ratifications all over the world.

Attaran A. proposal

Attaran A. and colleagues have proposed a global treaty to overcome substandard and falsified medicines problems with the help of comprehensive global strategy on which all stakeholders agree. They try to overcome the controversy over IP rights and confusion over terms.

A new global treaty could tackle both falsified and substandard medicines simultaneously and synergistically through legal, technical, and financial measures – it is more holistic approach to the problem.

They also proposed an action plan towards a global treaty. The treaty would need to fulfill five functions: to give clear terms, so as to avoid confusion and unwelcome over-reaches against legitimate medicines; define legally the different types of illegitimate medicines; define new public health crimes in international law, such as to manufacture, traffic, or sell falsified medicines; mandate intergovernmental cooperation so that countries report, investigate and prosecute transborder crimes and seize criminals' assets; create an ongoing intergovernmental forum to protect the legitimate medicines trade, such as by setting global standards for authenticating medicines with tracking and tracing technologies or by setting standards for medicine sales on the internet; include administrative provisions, particularly to give financial and technical assistance to strengthen medicine regulatory authorities in poorer countries (5).

CoE and UNODC are to reduce the legislative process into a regionalized political game, in which a small number of persons, mostly like-minded Europeans, function myopically and leave faults undetected that is the cardinal error as it saw Prof. Attaran (24). It follows a criminal law approach instead of a public health approach to address the issue of medicines with compromised quality, safety and efficacy without looking at the complexities of medicines regulation (25). Attaran also questioned the independence of the UNODC from the CoE. They worried the "unjustifiably criminalizing some generic medicines" (26).

Prof. Attaran proposed his own Model Law on Medicine Crime and it was published as a working draft, for review by the broader community, it tried to provide countries with a legislative template to fight medicine crime in their domestic law, putting the emphasis on public health where it belongs (27). Events in 2014 gave rise to doubts proposed by some scientists and representatives of international institutions, organizing and leading role, which could claim UNODC.

A similar situation has already been with IMPACT (2006–2008) that was replaced by WHO MSM (2012–2014), they were criticized for lack of transparency and progress, after that their work was terminated.

Mackey wrote that the advantage of Attaran's proposal is that it could also potentially address the "counterfeit" definition problem by attempting to legally define different types of illegitimate medicines by treating falsified (e.g., deliberate, intentional fraud of a criminal nature) and substandard (e.g., unintentional or negligence errors of regulatory nature) differently to avoid controversy (5). Mackey noticed that "most importantly, it would establish clear international binding rules and norms and if pursued would be only the second public health treaty after the WHO Framework Convention on Tobacco Control" (22).

CONCLUSION

The problem of preventing the counterfeit medicines entry into the world markets is multifaceted, and the authors must admit that the role of the international organizations in combating with counterfeit medicines was weak. During last 30 years, many international organizations tried to approach it from different directions, but the lack of mutual understanding, coordination and active engagement of all relevant stakeholders were the main reasons of the failure of the international community against this threat. WHO has been the main international health organization and it is reforming now, so the other organizations tried to fulfill this place and to address the issue.

There are many points for revision in the theme of counterfeit medicines.

The terms must be ordered worldwide for better understanding and effective struggle against this phenomenon. For this purpose "medicine" not "drug" for avoiding the confusion with narcotics (drugs) is proposed. The authors agree with the European Commission, that "falsified" not "counterfeit" is better as far as "counterfeit" refers to TRIPS convention and represents only IP aspect of the problem. Nonetheless, the MEDICRIME Convention uses the term "counterfeit".

The authors noticed there were three stages in counterfeit medicines combating: researching (1985–2005); discussing and trying to coordinate (2006–2015) and the stage of criminalizing – 2016 the MEDICRIME Convention entering into force. The stages separation depends on the form of activity of international organizations. In general, the activity of international organizations could be characterized as a matter of hit and miss and not a solution, but reaction to the organized crime activity.

The EU safety from counterfeit medicines was achieved due to the high level standards of all national regulatory affairs. The enhancing national regulatory affairs all over the world are the other possibility for preventing counterfeiting.

There were two scientific proposals on how a global partnership against the counterfeit medicines could be arranged:

1. Attaran – adoption of a global counterfeit medicine treaty;

2. Mackey – the establishment of an enhanced global health governance trilateral mechanism between WHO, UNODC, and Interpol to leverage the respective strengths and resources of these organizations.

Both of them were afraid of non-transparency and Europocentrism of the MEDICRIME Convention.

The third proposal will be realized – the MEDICRIME Convention highlights that international collaboration has to be intensified, national cooperation has to be optimized. The role of EDQM will increase.

The authors recognize that with the MEDICRIME Convention entering into force an absolutely new phase of counterfeit medicines combating is opened. Now there are clear notions of counterfeit, crimes in pharmaceutical sphere, states actions for accumulating and exchanging of information and effective fighting, etc. The CoE – a regional organization becomes the organization with a global influence through its treaties.

At the same time the Convention solves the problem only partially, since the agreement was made in an unrepresentative, nontransparent way. The lack of coherence in actions of stakeholders, and even, on the contrary, the presence of the difference between declared principles and their actions has been noticed. Thus, it hardly believed the Convention to become easily "successful" international treaty.

In this new situation, the authors recommend using the existing potential of international organizations, but according to the logic of the system development, it's only short-lived tactics. Therefore, all key stakeholders must continue to work on creating a new structure built on the democratic principles and the protection of public health.

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Conflict of interest

None declared.

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ADVERSE DRUG REPORTING IN COMMUNITY PHARMACY PRACTICE IN THE POMERANIAN DISTRICT IN POLAND

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Abstract: The changing role of the modern pharmacies involves reporting adverse drug reactions (ADR's). The aim of the study is to investigate the knowledge of community pharmacy professional staff (PS) about the new pharmacovigilance (PG) legislation and to analyze patients' attitudes towards the community pharmacy role in PG across north central region in Poland. Research material was collected in a group of pharmacy PS and patients from January 2014 to August 2014 in the Pomeranian District. A cross-sectional study was conducted. The research project consisted of three single anonymous surveys. Main outcome measure was knowledge of PS towards ADRs reporting, attitudes of patients towards ADRs in relation to PS in a community pharmacy. Over half of surveyed pharmacists (54.7%) and only 13% of pharmaceutical technicians know the correct and current definition of ADR. PS do not know what kind of ADRs should be reported - only 41% of pharmacists and 12.1% of technicians presented adequate knowledge. As far as the knowledge of where to notify is concerned - it was present in 60.7% of pharmacists and only 11.2% of pharmaceutical technicians. In most cases pharmacists (72%) know that patients can make their own notifications, technicians showed such knowledge only in 37.4% of cases. Patients who detect an ADR occurrence often do not transmit the information (19% of respondents). However, if they decide to report, they mostly talk to physicians (76.6%), and, less frequently (14.6%), to pharmacy PS. In the opinion of patients, however, the pharmacy PS (20.3%) are identified as subjects involved in reporting ADRs and those who can transmit information on the ADRs. The results suggest that pharmacists' knowledge of the rules of reporting ADRs is superior to that of pharmaceutical technicians. In fact, PS are not sufficiently prepared for reporting ADRs. Patients, in turn, do not have adequate awareness of reporting the observed ADRs. They, however, associate the community pharmacy with PG system, although the extent is unsatisfactory. In this regard, a more effective training of PS would be recommended along with tailored social campaigns to inform about the PG system in Poland.

Keywords: adverse drug reaction's reporting, pharmacovigilance, pharmacy law, community pharmacists, consumer reporting

Impact of findings on practice
- It is necessary to improve the level of knowledge of the principles of reporting ADRs among the professional staff of community pharmacies.
- It is also necessary to consolidate public awareness on the reporting of ADR and the role of community pharmacies in pharmacovigilance system.

The guarantee of medicinal product (MP) safety is an important element of the health care system. In this system, it is extremely important to report the observed adverse reactions that can help verify existing drug safety information. Voluntary adverse drug reaction (ADR) reporting is one of the most versatile pharmacovigilance (PG) methods, because, among other advantages, it covers the entire population as well as all drugs throughout their commercial life (1). PG relates to the detection, assessment, understanding and prevention of ADR's and other drug related problems (2).

In developed countries pharmacists from community pharmacies (CP) play a significant role in PG as they make a lot of ADR reports of adequate quality (3, 4). In the Netherlands, approx. 40% of reports are made by pharmacists from CPs. Similarly, in Australia - also approx. 40% of reports are sent by CPs. In Spain, this percentage is approx. 25%, in Japan - 39%, but the above figures include the activities of pharmacists from both hospital and CPs. Up to approx. 88% and 68% of reports in Canada and USA, respectively, are made by phar-

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macists, but the data relate to hospital pharmacists (5). Pharmacists from CPs mainly report OTC ADR, as well as adverse reactions identified by patients, e.g., skin lesions (5-8). The study also demonstrated that in the countries where pharmacists actively participate in the system of PG, they view reporting as a standard professional duty (4, 9). Studies conducted among physicians indicated, however, that according to 95% of subjects, the involvement of the pharmacists in the detection and reporting of ADR is very much needed, which means that doctors do not negate the role of pharmacists (10).

The number of reports of ADRs in Poland is small, as illustrated in Table 1. Pharmacists only make several reports per year (11).

Polish pharmacists act in compliance with their statutory duty to report ADR's (section 5b of the Law on Pharmaceutical Chambers). The transfer of this information, is - in particular - the task of the pharmacy manager, pursuant to the Pharmaceutical Law (PL) section 88 article 5 point 3. Polish pharmacy staff also includes pharmaceutical technicians (the qualification is obtained after completing a 2-year post-secondary school). Their exact number is not known because they are not required to register and they do not have a professional self-governing body, unlike pharmacists. In the current economic situation of Polish pharmacies a lot of pharmaceutical technicians are employed to substitute the more expensive work of pharmacists. Technicians obtained the opportunity (legal right) to report ADRs in 2013. Reporting is voluntary. In 2013, Polish patients were granted the right to report ADRs on their own to marketing authorization holders (MAH) or to the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products (URPL), which record all instances of ADRs on Polish territory. Patients can also provide information about ADRs to medical professionals (including PS in a CP). In the first year of operation of the new rules, i.e. in 2014, patients reported only 30 ADRs.

In 2013, the narrow definition of ADRs was changed to account for the commonly applied dose and a broader definition was introduced. Currently, any adverse and unintended effect of the drug is an ADR, irrespective of the dose (section 2 point 3 of the PL). The date of filing is not specified.

A severe adverse reaction to a MP is defined as an action that results in death of the patient, a life-threatening event, hospitalization or its extension, persistent or significant bodily injury or any other effect of a MP, which the doctor deemed serious to his or her knowledge or a disease, congenital defect or damage to the fetus, regardless of dose MP (section 2 point 3d, PL). Medical professionals (including pharmacists and technicians) are obliged to report serious adverse reactions to a MPs within 15 days of being informed of their occurrence (section 36f, PL).

Table 1. Number of spontaneous notifications to the President of the Office of Registration of Medicinal Products (URPL) in 2008-2012 before the PG system reform (11).

Year	Number of spontaneous reports
2008	904
2009	1211
2010	811
2011	1030
2012	1144

Reports are to be made on a form available on the website of the Office for URPL and sent by mail, e-mail or fax.

There is a lack of studies that address the knowledge of ADR reporting in pharmacy PS and attitudes of patients in the event of ADR, particularly in relation to pharmacies PS. This project was initiated by the need for the better understanding of the role of pharmacies in PG. Understanding the above role and the state of ADR knowledge among PS can help change the currently insufficient level of reporting, taking into account that underreporting is related with knowledge represented by health professionals (12).

EXPERIMENTAL

The aim of the study is to help understand the role of pharmacies in the system of PG in the situation of a very low level of ADR reporting and evaluate the preparation of pharmacy PS for such reporting. The above aim was sought by analyzing:

- the state of legal knowledge of pharmacists and pharmaceutical technicians regarding the principles of ADR report submission and
- the attitudes of patients in a situation of detecting an ADR.

The Ethics Committee of the Medical University of Gdansk does not require consent, or give opinions, for this type of survey research.

A cross-sectional study was conducted. The research project consisted of three single anonymous surveys in employees of pharmacies: pharmacists (Part A) and pharmaceutical technicians (Part B), and patients who attend pharmacies (Part C of

the project). The study research tool was a questionnaire survey, designed on the basis of the research team's discussions and literature review. The study was conducted in an open, voluntary and confidential manner. We used an environmental questionnaire. The participants of the tests included pharmacy PS and patients of the Pomeranian pharmacies approved by the provincial pharmaceutical inspector for possible cooperation with the Medical University of Gdansk (the only higher school which educates pharmacists in the Pomeranian District) that agreed to take part in the study. The research process was preceded by a pilot study conducted in three groups of respondents (A, B and C). Before the test, the consent for the study conduct was obtained from owners, managers of pharmacies and participants.

A trained interviewer distributed the survey in pharmacies. The respondents could put the completed questionnaire in a special box or hand it to the researcher. The sample selection was purposeful - pharmacists and pharmaceutical technicians working in selected public pharmacies were respondents in Parts A and B, respectively. In Part C, clients of health care services, using medications and visiting selected pharmacies.

Questionnaires (450) were distributed to pharmacy employees for each professional group. Pharmacists obtained 232 completed forms (51.6% response rate) and from pharmaceutical technicians - 107 (23.8% response rate). Two thousand (2000) printed questionnaires were distributed among patients; 635 properly completed surveys were collected, thereby obtaining the response rate at 31.8%.

The study was conducted from January 2014 to August 2014 in the Pomeranian District. Each survey questionnaire was tested on 10 respondents under the same conditions in which the final questionnaire would be conducted. Small changes were made with regard to the order of items and phrasing of text.

Table 2. Pharmacists and pharmacy technicians survey groups characteristic.

		Pharmacists n = 232	Pharmacy technicians n = 107
Age			
	under 39	123 (53.0%)	56 (52.4%)
	40-60	103 (44.4%)	44 (41.1%)
	over 60	6 (2.6%)	7 (6.5%)
Professional experience (years)			
	up to 10	107 (46.1%)	70 (65.4%)
	11-30	105 (45.3%)	30 (28.1%)
	more than 30	20 (8.6%)	7 (6.5%)
Professional position			
	manager	106 (45.7%)	0
	counter staff	126 (54.3%)	107 (100%)
Specialization			
	lack	133 (57.3%)	107 (100%)
	holder	99 (42.7%)	0
Pharmacy profile			
	in outpatient clinic	67 (28.9%)	41 (38.3%)
	in shopping center	40 (17.2%)	23 (21.5%)
	residential	125 (53.9%)	43 (40.2%)
	network pharmacy	131 (56.5%)	59 (55.1%)
	independent pharmacy	101 (43.5%)	48 (44.9%)
	in small town	92 (39.6%)	38 (35.5%)
	urban	102 (44.0%)	54 (50.5%)
	rural	38 (16.4%)	15 (14.0%)

Survey forms consisted of two parts. They contained the questions on particulars characterizing the test group and the main questions. All questions were non-projective and were addressed directly to the respondent. Survey forms were accompanied by information on the purpose of the study, individual researcher and the researcher's institution. They also contained instructions for respondents. Parts A and B of the questionnaire had 9 closed-ended questions in the content-related section. One of them concerned a subjective assessment of the degree of knowledge on ADR reporting. It used balanced scale. The next 8 questions verified actual knowledge of the rules of proper reporting. The content-related section of the questionnaire addressed to the patients (C) consisted of 6 closed-ended questions and one half-open question. Two filtering questions were used. Four times cafeteria questions were used. The questionnaires were examined in linguistic and interpretative terms. The questionnaires were designed in such a way that the answers were not mutually exclusive.

All statistical calculations were performed with the use of a statistical package - StatSoft. Inc. (2011). STATISTICA (data analysis software system), version 10.0. www.statsoft.com. and an Excel spreadsheet. χ^2 independence test was used for categorical variables, the level of significance was set at $p \leq 0.05$ for all the calculations.

RESULTS

Characteristics of the study group

In the group of surveyed pharmacists (n = 232) women predominated (90.5%). The study group of pharmaceutical technicians (n = 107) also mainly comprised women (98.1%) which is a specific feature of pharmaceutical PS. Survey groups (A, B) characteristics is illustrated in Table 2.

Patients study group (part C) characteristics is illustrated in Table 3.

Table 3. Patients survey group demographic characteristics.

Patients (n = 635)		
Age		
	up to 39	259 (40.8%)
	40-60	261 (41.1%)
	above 60	115 (18.1%)
Gender		
	female	461 (72.6%)
	men	174 (27.4%)
Residence		
	small town	233 (36.7%)
	urban	277 (43.6%)
	rural	125 (19.7%)
Education level		
	basic	70 (11.0%)
	high school	343 (54.0%)
	academic	222 (35.0)

Knowledge of pharmacy PS on ADR reporting

In the study A 31.4% of respondents rated their knowledge as good, 30.2% - as average, and 28.9% - as poor. Very good knowledge of the rules for reporting ADRs was indicated by 8.6%. Only 2 pharmacists (0.9%) rated their knowledge as very poor. In terms of specialization, the study demonstrated that pharmacists-specialists statistically more frequently declared good knowledge of ADR reporting (χ^2 value = 14.13, p-value = 0.0068). It was also noted that pharmacists from independent pharmacies assessed their knowledge of the rules for ADR reporting as good more frequently than pharmacists from network pharmacies (χ^2 value = 15.81, p-value = 0.0452). Technicians rated their knowledge on ADR reporting in the following way: 67 people (62.6%) poor, 31 people (29%) - average, 5 respondents (4.7%) - very poor and 4 respondents (3.7%) - good.

Participant-pharmacists (54.7%) and 13% technicians were able to provide the correct and current legal definition of an ADR. An incorrect answer (reaction after applying the usual dose) was quite frequently provided (35% in A and 64% in B), indicating a lack of current knowledge. The answer 'I do not know' was given by 10.3% respondents in group A and by 13% in group B.

In the group A (52%) and in group B (53.3%), more than a half of respondents could not properly identify ADRs that should be reported giving an answer: 'only a serious adverse reaction' or 'just an unspecified adverse reaction'. The correct answer was chosen only by 41% in A and by 12.1% in B group. Seven percent of A and 34.6% of B respondents declared lack of knowledge on the subject.

In the A group the right answer to the question on who should report an ADR was marked by 60.7% of respondents and in group B by 11.2%. Very few respondents erroneously indicated that the information has to be transmitted to the Pharmaceutical Inspection – (2.2% in A and 26.9% in B) or National Health Fund (5.2% in A and 24.0% in B). Of group

A 31.9% and 37.9% of group B respondents admitted they did not know to whom they need to ADR's admitted.

Pharmacists in most cases (79.3%) were able to correctly indicate that an ADR report must be made on a special form. Only 2.4% of respondents considered it appropriate to notify by telephone, while 9.6% took the view that a notification in writing will be sufficient but without the use of a special form and 8.7% pharmacists showed lack of knowledge. The obtained results also show that managers of pharmacies (in charge of reporting ADRs) to a statistically significant extent most frequently knew that the ADR report is to be made on a special form (χ^2 value = 10.04, p-value = 0.0397). The participant-technicians were mostly convinced that the application can be made in any written form (41.9%), by telephone (4.8%) and only 39.3% of respondents provided the right answer. Lack of knowledge was revealed in 14% of respondents.

Respondents (58.2%) in A and 36.4% in group B showed knowledge of the rules of completing an ADR form and providing only patient's initials. Respondents also marked the responses such as:

Table 4. Distribution of correct answers to the questionnaire on knowledge ADR reporting addressed to pharmacists and pharmaceutical technicians.

Statement	Pharmacists (n = 232)	Pharmacy technicians (n = 107)
ADR is any unfavorable and unintended drug effect	127 (54.7%)	14 (13.0%)
All ADRs should be reported	95 (41.0%)	13 (12.1%)
Information on ADRs transmitted by the patient should be reported to URPL and MAH	141 (60.7%)	12 (11.2%)
ADR reports should be made on a special form	184 (79.3%)	42 (39.3%)
In an ADR report only patient's initials are provided	135 (58.2%)	39 (36.4%)
Current regulations do not define the deadline for ADR reports from the date of acquiring information	45 (19.5%)	17 (15.0%)
Severe ADRs should be reported within 15 days from the date of acquiring information	3 (1.3%)	2 (1.9%)
The patient (or her/his statutory representative) can independently report an adverse reaction to URPL or MAH	167 (72%)	40 (37.4%)

Table 5. Relationship between age and the respondents' conviction that the information on ADR should be notified to a PS in a community pharmacy - study C.

To whom do you think a patient should report any occurrence of side effects of a medicine?		Age			Number of answers in total
		up to 39 years (n = 258)	40-60 years (n = 261)	over 60 years (n = 114)	
A patient should notify pharmacy PS	Number	65	47	17	129
	Percentage (%)	25.2	18.0	14.9	

'provide the name and address of residence' (9.6% A and 13.5% B); 'provide the statistical social security number' (12.4% A and 8.6% B); 19.8% of pharmacists and 41.5% of technicians admitted lack of knowledge on this issue.

When asked about the deadline for an ADR report when the notification is made by a patient in the pharmacy, pharmacists and technicians responded intuitively ('immediately') – 64.7% in A and 68.3% in B group. The correct answer was provided by 19.5% of A respondents and 15% of B, 15.8% of A and 16.7% of B respondents admitted they did not have knowledge on the issue. In the group of specialist-pharmacists, the correct answer was provided more frequently (χ^2 value = 10.22, p-value = 0.0168). When asked about legally set deadline for the serious adverse reaction report, the surveyed pharmacists and pharmaceutical technicians most often replied 'immediately' (94.2% and 86.6%, respectively) or 'the law does not specify a time limit' – 2.7% and 7.6%, respectively, and 'I do not know' (1.8% and 3.9%, respectively). The correct answer was granted by only 1.3% of pharmacists and 1.9% of technicians.

Most surveyed in A group (72%) knew that patients can make an ADR report to the URPL or to MAH, which contrasts with technicians, of whom only 37.4% knew about this fact. The answer 'I do not know' was given by 13.3% of pharmacists and 32% of technicians. An incorrect answer was provided by 14.7% in A and 30.6% in B group.

Distribution of correct answers to the questionnaire on knowledge ADR reporting addressed to pharmacists and pharmaceutical technicians is illustrated in Table 4.

Most often, the correct answer was chosen by those respondents people who declared good knowledge of the reporting of ADRs. In contrast, respondents evaluating their knowledge as a quite good most frequently provided an incorrect answer (χ^2 test results indicated a statistically significant relationship: the value of the test statistics $\chi^2 = 22.96$, p-value = 0.0034).

Table 6. Reasons for selecting of pharmacy PS from other health professionals by patients providing information on ADRs (n = 129)*.

Reason for selecting	
Pharmacy PS	Participants
Easy access	96 (74.4%)
Confidence	80 (62.0%)
Expertise	68 (52.7%)
Advisor	43 (33.3%)

*The answers do not add up to 100% as the participants could select more than one answer.

Attitudes of patients

Part C of the study demonstrated that 21.6% (137) of patients observed the ADR of treatment while applying medications during the last year. Hundred and five (76.6%) of respondents (n = 137) reported it to their physicians and 26 respondents (19.0%) did not communicate this information to anyone (respondents could choose multiple answers). Respondents also informed a PS in a CP (20 respondents; 14.6%), nurse (4 respondents; 2.9%) and a MAH (3 persons; 2.2%). None of the respondents provided a notification to the URPL. Patients (n = 137) mainly observed ADRs of prescription drugs (97.8%) and only 3 people recorded ADRs after taking an OTC drug. ADR were mainly reported in an informal oral form (92%), rarely in writing (8%).

When asked about their preferences concerning their opinion should report the occurrence of ADR (it was possible to select multiple answers) the most (600 – 94.5%) indicated that the physician; 129 (20.3%) – PS in a CP, and 49 patients (7.7%) – the nurse. In the opinion of 22 patients (3.5%) there is no need to report ADR at all. The distribution of answers to this question depended on age to a statistically significant extent (the value of $\chi^2 = 6.69$, p-value = 0.0353). Respondents under 39 years of age significantly more frequently responded that an ADR should be reported to a PS in a CP.

Relationship between age and the respondents' conviction that the information on ADR should be notified to a PS in a community pharmacy is illustrated in Table 5.

Patients who would report an ADR to the PS of a pharmacy (n = 129) and no other health professionals indicated that they had confidence in them (62%), recognized them as professionals knowledgeable in medications (52.7%) treated them as advisers (33.3%) or appreciated the easy access to their services (74.4%) – it was possible to select multiple answers. The answer 'none of the above' was marked by 4 people, who, however, did provide their own answers.

Reasons for selecting of pharmacy PS from other health professionals by patients providing information on ADRs are illustrated in Table 6.

Patients also answered the question concerning who (which healthcare professional) informs them of ADRs. It was shown that most often it is the physician (86.7%), PS in a pharmacy (54.4%), nurs-

es (43.2%) - it was possible to select multiple answers.

DISCUSSION AND CONCLUSIONS

We performed a cross-sectional questionnaire-based assessment of pharmacists' and technicians' knowledge on ADR reporting and patients' attitudes towards ADRs in relation to PS of a CP in the north-central region of Poland. The possible Hawthorne effect may be pointed out as a limitation for the focus group. Another possible limitation of the study is also the possibility of guessing answers by risk taking respondents.

Our results have revealed that the PS have insufficient knowledge on ADR reporting. Previous foreign studies documented a similar lack of knowledge in other countries.

Pharmacists can make a major positive contribution to the quality and number of reported ADRs (13-15). The role of professional pharmacists in the system of PG is the subject of numerous studies (1, 10, 14, 16-22). They point to the underlying causes of not undertaking to make spontaneous notifications. It has been proven that most frequently pharmacists do not have a systematic knowledge of legal regulations of reporting, which prevents submitting a formally correct notification (20, 22). Pharmacists also do not know how to obtain notification forms (15, 17, 22, 23).

Just over a half of the surveyed pharmacists (54.7%) could correctly produce the current definition of ADR. In a study conducted in Turkey, an even smaller group - 26% of pharmacists (22) knew the definition. However, the question in the current study had a different format from that used in the previous study, which makes a direct comparison difficult. In China, in a survey of hospital pharmacists, 69.5% of respondents knew the correct definition (24). In the group of pharmacists, in spite of declaring the good knowledge of the rules for ADR reporting, more than half of respondents (52%) could not correctly identify reactions that should be reported. For comparison, in a study conducted in Turkey, only 18.3% of pharmacists showed an incorrect understanding of this topic (22). However, in the study by Su et al., 35% of hospital pharmacists indicated that all ADRs should be reported (24). In Oman, pharmacists surveyed (20%) were convinced that only the ADRs which concern new drugs should be reported (25). As indicated by research, pharmacists are quite often not aware of where and to whom they should report ADRs. In Oman, only 29% of respondents showed adequate knowledge on the subject (26), in this study, pharmacists answered correctly in 60.7% of cases. The surveyed pharmacists in most cases (79.3%) knew that the report must be made on a special form. In a study in Iran only 24.4% respondents showed such knowledge (15), and in Nigeria – 12% (20). In the study group, most pharmacists (72%) knew about the possibility of ADR reporting by the patient himself or herself. For comparison, in Portugal 69.5% of respondents knew about those issues (27).

Very few studies include knowledge of pharmaceutical technicians on reporting ADRs. Research by Abdel-Latif revealed that 61% of technicians working in hospital pharmacies are aware of the national system of PG (26). No research papers on CPs were found.

In the study 21.6% of patients admitted they had observed ADRs, and none of the respondents reported this fact to URPL, the registrar for ADRs. In Portugal, it was shown that 57.6% of respondents suffered from an ADR and only one person reported this fact to the National Pharmacovigilance System (28). In our study, patients, who observed ADRs contacted the PS in a CP only in 14.4% of cases. Typically, they informed their physician about the situation (76.6%). A study conducted in Portugal showed that patients reports ADRs to a physician in 35.9% of cases and in 33.7% of cases - to PS in pharmacy (28). Some studies indicate that patients are motivated to report ADR to the data collection bodies because it is difficult to discuss the ADR with a medical practitioner or pharmacist (29). A patient usually learns about ADRs from a medical practitioner (86.7%), less frequently - from a PS in a pharmacy (54.4%). The study conducted by Abdel-Latif demonstrated similar results - that medical practitioners give advice in 82.4% of cases. The share of pharmacists in this kind of advising is higher - it amounted to 75.7% (26).

The results of the study indicate that age or years of experience in CP do not influence the level of professional staff knowledge on ADRs and are consistent with previous studies from Norway (17) and Turkey (22). No relation was shown between the results and the nature of workplace.

The results concerning the legal expertise of pharmacists in reporting ADRs allow us to conclude that it should be explored. Legal knowledge on ADRs reporting among surveyed pharmaceutical technicians is, in turn, poorly established and insufficient. The results indicate that the test group is not adequately prepared to implement the patient right in the pharmacy. Training of the PS of pharmacy is therefore recommended.

In the cases of ADRs, patients usually report them to a physician, however, about 20% of the respondents believe that the occurrence of side effects should be reported to the PS of a pharmacy. This reflects the recognition of the role of the pharmacy as a place in which to report ADRs, but unfortunately, only in a small proportion of the studied community. What is disturbing is the total amount of information which fails to be transferred, which indicates the low awareness of patients in this regard. The role of pharmacies should therefore be to educate patients in the area of ADRs.

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SHORT COMMUNICATION

PHYTOCHEMICAL, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *PTERIS CRETICA* L. (PTERIDACEAE) EXTRACTS

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The antibiotics resistance in microbes gives emphasis to explore new potent and promising drug molecules from natural sources (1). In this connection, natural plant products, being safe, reliable, least toxic and economical than the available synthetic drugs, have been abundantly used (2). Therefore, plants can be used extensively to explore new effective and therapeutically potent antibacterial agents (3-5).

The whole part of *Pteris cretica* fern, especially young frond, is used in Chinese traditional medicines as antipyretic, antidote and burn treatment (6). It is also used as antimicrobial on wound healing in the form of fronds paste (7).

Many pathological processes involve free radicals or these may be released during pathological stages (8-11). Some serious diseases like Parkinson, Alzheimer, atherosclerosis, heart attacks and chronic fatigue system have been found connected with the involvement of free radicals (11-16).

Due to susceptibility of carcinogenic effect the use of synthetic antioxidants like butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been restricted in food stuff (17). Therefore, efforts for exploration of natural antioxidants, especially of plant origin, have been increased tremendously in recent years (18).

The present investigation has been carried out to evaluate *Pteris cretica* extracts for phytochemical analysis, antimicrobial and antioxidant activities.

MATERIALS AND METHODS

All solvents and regents used were of analytical grade. Ciprofloxacin, methicillin and clotrimazole were collected from Drug Testing Laboratories (DTL), Lahore, Pakistan. Spectrophotometer (Analytik Jena, Spekol 2000), weight balance (Shimadzu BL220H), incubator (Memmert IN110) and hemocytometer were used.

Collection and identification of plant materials

The shoots along with leaves (fronds) of plant were collected from the Azaad Kashmir area in the month of April and identified by Prof. Dr. Zaheer-ud-Din Khan at the department of Botany, Govt. College University (GCU), Lahore, Pakistan. A voucher specimen of *Pteris cretica* L. (voucher no. 2142) was deposited in the Herbarium of GCU. The collected plant material was dried under shade to avoid enzymatic degradation and fungal growth.

Extraction

One kg of dried plant material was pulverized and extracted in n-hexane, chloroform and ethanol successively using Soxhlet apparatus (19). The extraction process with each solvent was continued for 7-14 days until the solvent in thimble chamber of Soxhlet appeared colorless. These extracts were filtered and dried on rotary evaporator at about 40°C under reduced pressure to get resinous extracts of n-

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hexane, chloroform and ethanol with 11%, 4.4% and 17.35% yields, respectively, (with respect to the starting dry plant mass).

Phytochemical analysis

The dried extracts were subjected for the evaluation of major secondary metabolites using the standard qualitative methods with little modification (20). These tests showed presence of terpenoids, sterols, flavonoids, alkaloids, saponins, tannins and carbohydrates.

Microorganisms

Seven pathogenic Gram positive (*Bacillus subtilis* (ATCC 11774), *Salmonella typhi* (ATCC 14028), *Staphylococcus aureus* (ATCC 12600), *Micrococcus luteus* (ATCC 10240), *Clostridium sporogenes* (ATCC 19404), *Pasteurella multocida* (ATCC 12945) and *Sarcina lutea* (ATCC 9341)) and three Gram negative (*Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), and *Klebsiella pneumoniae* (ATCC 13882)) bacterial strains along with three fungal strains (*Candida albicans* (ATCC 10231), *Aspergillus oryzae* (ATCC 10124) and *Fusarium saloni* (ATCC 11712)) were obtained from Pakistan Council of Scientific and Industrial Research (PCSIR) laboratory, Lahore, Pakistan.

Fresh inoculums were prepared by taking a loopful of bacteria from stock culture and antiseptically transferred to sterile nutrient broth medium which was incubated at 37°C for 24 h. For maintaining fungal cultures, 3.9% aqueous diphenic acid (DPA) (pH 5.6) was utilized. Fungal slant (3-5 days old) was taken as an inoculums and spore suspension was developed by the addition of 10 mL sterilized 0.05% (w/v) monoxal OT in the inoculum. From this fresh spore suspension, 1 mL was cultured in about 25 mL of cultivation medium. Spores were quantified by hemocytometer.

Determination of antibacterial activity

For determination of antibacterial activities, dried extracts of *Pteris cretica* L. in n-hexane, chloroform and ethanol were tested against ten pathogenic bacterial strains using the agar well diffusion assay.

Each fresh bacterial culture (about 10^5 to 10^6 cells/plate) was inoculated in the molten nutrient agar plate. After solidification, about 6 wells with 8.00 mm diameter were bored with the help of a sterilized stainless steel cork borer in each solidified agar plate. Three concentrations (5, 50 and 100 mg/mL) of each plant extract were prepared in the gum acacia solution (4.5%). About 100 µL of every concentration was taken to load each well. In this way all Petri dishes with specific bacteria were loaded by different concentrations of each extract. The central well in each plate was filled with 100 µL of gum acacia solution (4.5%) as a negative control. Two antibiotics: ciprofloxacin and methicillin were used as standards or positive control by pouring into two separate wells in each Petri dish. The plates were incubated at 37°C for 24 h and the zone of inhibition was measured in millimeters. The experiment was repeated in six replicates and average activity was calculated (21).

Determination of antifungal activity

Extracts were tested against fungal strains and zone of inhibition were measured in a similar way as described in the antibacterial activity.

Determination of antioxidant activity

Radial scavenging activity using DPPH method

The radical scavenging activity of different extracts of *Pteris cretica* L. was determined using DPPH assay (22). The dried plant extracts were diluted in methanol (1 mg/mL). 0.1 mL of each dilution was taken in separate test tubes and mixed with 3 mL of 0.1 mM methanol solution of DPPH. In the same way standard solution (ascorbic acid) was prepared in a separate test tube.

The control was prepared by mixing of 0.1 mL methanol with 3 mL of 0.1 mM methanol solution of DPPH. After vigorous mixing, all these test tubes were kept at room temperature for about 30 min.

Methanol utilized in the preparation of dilutions, was also used for the baseline correction. The absorbance of all these samples was measured at 588 nm using the UV spectrophotometer. The percentage radial scavenging activity (%RSA) was measured using following formula and the experiment was repeated in triplicate.

$$\%RSA = \frac{A_c - A_s}{A_c} \times 100$$

where A_c = absorbance of sample and A_s = absorbance of control.

β-Carotene-linoleate model

The antioxidant activity of *Pteris cretica* L. extracts was also determined using the β-carotene-linoleate model system (23). β-Carotene (0.2 mg) was dissolved in 0.2 mL chloroform in small round bottom flask and linoleic acid (20 mg) and Tween 40 (polyoxyethylene sorbitan monopalmitate) (20 mg) were added. After proper mixing, the chloroform was removed on rotary evaporator at 40°C under

vacuum. Distilled water was added to the resulting mixture to make up final volume of 50 mL. From this emulsion, 4 mL aliquots were pipetted into different test tubes having 0.2 mL of each *Pteris cretica* L. extract (1 mg/mL ethanol). For standard, 0.2 mL BHT (1 mg/mL ethanol) was added to 4 mL emulsion. Likewise a control was prepared by mixing of 0.2 mL ethanol with 4 mL emulsion (mentioned above). All these test tubes containing samples, standard and control were kept at 50°C in a water bath and the absorbance at 470 nm was taken at zero time (t = 0 min). Then, after an interval of 15 min, the absorbance of materials in all test tubes was measured at 470 nm continuously until the color of β-carotene disappeared in control test tube (usually t = 120 min). A mixture, prepared in a similar way as mentioned above but without β-carotene, served as blank. The experiment was conducted in triplicate.

Table 1. Phytochemical assessment of constituents of Pteris cretica L.

Groups	Phytochemical tests	Results
1) Alkaloids		
	i) Mayer's test	-
	ii) Wagner's test	-
	iii) Hager's test	-
	iv) Dragendroff's test	++
2) Carbohydrates		
	i) Molish's test	+++
	ii) Barfoed's test	+
	iii) Benedict,s test	+++
3) Protein and amino acids		
	i) Biuret test	+++
	ii) Ninhydrin test	-
	iii) Millon's test	-
	iv) Xanthoprotic test	-
4) Flavonoids		
	i) Ferric chloride test	+++
	ii) Alkaline reagent test	+
	iii) Lead acetate test	+++
5) Saponins		
	i) Foam test	+
	ii) Bromine water test	-
	iii) Legal's test	-
6) Steroids		
	i) Salkowski's test	++
	ii) Liebermann's test	++
	iii) Sulfur test	+++
7) Terpenoids		
	i) Salkowski's test	+
	ii) Liebermann's test	++
8) Lipids		
	i) Soap formation test	-
9) Tannins		
	i) Ferric chloride test	+++

- = absent, + = weakly present, ++ = moderately present and +++ = strongly present.

Table 2. Antimicrobial activities of different concentrations (5, 50 and 100 mg/mL) of n-hexane, chloroform and ethanol whole plant extracts of *Pteris cretica* L.. Each value represents the mean ± standard error of diameter of zone of inhibition (mm) of six replicates.

***Pteris cretica* L.**	**Bacterial strains**										**Fungal strains**		
	Gram positive							**Gram negative**					
Extracts/Drug (Conc.)	***Bacillus subtilis***	***Salmonella typhi***	***Staphylococcus aureus***	***Micrococcus luteus***	***Clostridium sporogenes***	***Pasteurella multocida***	***Sarcina lutea***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Klebsiella pneumoniae***	***Candida albicans***	***Aspergillus oryzae***	***Fusarium saloni***
n-Hexane													
5 mg/mL	10.00 ± 0.00	-	09.25 ± 0.42	-	-	-	-	-	-	-	11.00 ± 0.50	10.00 ± 0.00	-
50 mg/mL	12.00 ± 0.39	-	-	-	-	-	-	-	-	-	12.55 ± 0.38	-	-
100 mg/mL	13.00 ± 0.46	-	-	-	12.00 ± 0.39	-	-	-	-	-	18.00 ± 0.00	-	-
Chloroform													
5 mg/mL	-	-	11.50 ± 0.43	-	-	-	-	-	-	-	-	-	-
50 mg/mL	11.33 ± 0.39	-	12.00 ± 0.00	-	11..00 ± 0.41	-	-	-	-	-	-	12.00 ± 0.39	
100 mg/mL	13.67 ± 0.24	-	10.00 ± 0.00	-	14.00 ± 0.00	-	-	-	-	11.75 ± 0.22	14.50 ± 0.25	-	-
Ethanol													
5 mg/mL	-	-	08.00 ± 0.00	-	-	-	-	-	-	-	10.50 ± 0.25	-	-
50 mg/mL	14.00 ± 0.35	-	10.00 ± 0.00	-	09.00 ± 0.40	-	-	09.33 ± 0.24	-	09.50 ± 0.25	12.71 ± 0.23	-	-
100 mg/mL	-	-	12.25 ± 0.22	-	12.00 ± 0.00	-	-	09.67 ± 0.23	-	12.00 ± 0.00	13.75 ± 0.22	-	-
Methicillin (1 mg/mL)	26.66 ± 0.36	23.33 ± 0.23	23.66 ± 0.23	25.33 ± 0.46	20.00 ± 0.00	24.00 ± 0.00	-	24.33 ± 0.23	20.66 ± 0.23	20.66 ± 0.23			
Ciprofloxacin (1 mg/mL)	28.33 ± 0.33	24.33 ± 0.23	26.33 ± 0.21	20.66 ± 0.23	28.00 ± 0.00	28.00 ± 0.00	29.00 ± 0.00	24.66 ± 0.33	22.33 ± 0.23	20.33 ± 0.36			
Clotrimazole (1 mg/mL)											46.66 ± 0.36	19.00 ± 0.00	46.33 ± 0.36

All values were significantly different as $p < 0.05$; – = no effect.

The percentage antioxidant activity (%AA) of *Pteris cretica* L. extracts was determined in terms of bleaching of β-carotene using the following formula:

$$\%AA = 100 \times [1 - \frac{A_s^0 - A_s^t}{A_c^0 - A_c^t}]$$

where A_c^0 = absorbance of control at t = 0 min, A_s^0 = absorbance of sample at t = 0 min, A_c^t = absorbance of control at t = 120 min and A_s^t = absorbance of sample at t = 120 min.

RESULTS

The qualitative phytochemical analysis of *Pteris cretica* L. extracts revealed the presence of flavonoids, sterols, tannins, carbohydrates, saponins, alkaloids and proteins as presented in Table 1.

Present investigation is the first report on antimicrobial activity of *Pteris cretica* L. extracts. The results of antibacterial activity (zone of inhibition) of various crude extracts (n-hexane, chloroform and ethanol) of *Pteris cretica* L. against seven Gram positive species and three Gram negative species have been shown in Table 2. The antimicrobial activity against all these strains is judged by the zones of inhibition as compared to standard antibiotics, i.e., ciprofloxacin and methicillin. n-Hexane extract of *Pteris cretica* L. at different concentrations (5, 50 and 100 mg/mL) did not show any activity against *Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi, Micrococcus luteus, Pasteurella multocida* and *Sarcina lutea*. It showed significant results against *Bacillus subtilis, Staphylococcus aureus* and *Clostridium sporogenes*.

Chloroform extract at any of the used concentrations did not display antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhi*, *Micrococcus luteus*, *Pasteurella multocida* and *Sarcina lutea*, whereas it displayed zones of inhibition against *Bacillus subtilis, Staphylococcus aureus, Clostridium sporogenes* and *Klebsiella pneumoniae* at various concentrations as depicted in Table 2. Likewise, ethanol extract also showed activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Clostridium sporogenes*, and *Klebsiella pneumonia*.

n-Hexane, chloroform and ethanol extracts at different concentrations (5, 50 and 100 mg/mL) did not present zones of inhibition against *Fusarium saloni* but displayed zones of inhibition against *Candida albicans* and *Aspergillus oryzae* as mentioned in Table 2.

Table 3. Antioxidant activity of *Pteris cretica* L. extracts using DPPH and ß-carotene methods. Each value of mean is average of three repeated experiments ± standard error (S.E.).

Pteris cretica L.	DPPH activity		ß-Carotene activity	
Extracts/Standard	% RSA	Mean % RSA ± S.E.	% AA	Mean % AA ± S.E.
n-Hexane	61% 60% 60%	60.33 ± 0.27	62.80% 63.00% 63.50%	63.10 ± 0.17
Chloroform	67% 67% 68%	67.33 ± 0.27	64.60% 64.10% 64.90%	64.53 ± 0.19
Ethanol	66% 65% 65%	65.33 ± 0.27	67.80% 68.20% 67.90%	67.97 ± 0.12
Ascorbic acid	86% 86% 84%	85.33 ± 0.34		
BHT			88.90% 89.10% 89.00%	89.00 ± 0.00

All values were significantly different as $p < 0.05$.

Pteris cretica L. has been investigated the first time for antioxidant evaluation in present study. Potent DPPH and β-carotene antioxidant models were used to check antioxidant potential of *Pteris cretica* L. extracts.

The scavenging potential of the plant extract can be estimated by the degree of discoloration (of DPPH). n-Hexane, chloroform, and ethanol extracts of *Pteris cretica* L. and standard ascorbic acid showed 60%, 67%, 65% and 85% free radical scavenging activity (%RSA), respectively. The results are tabulated in Table 3.

The percentage antioxidant activity (%AA) of *Pteris cretica* L. was also estimated by bleaching of β-carotene. Results are evident that n-hexane, chloroform, and ethanol extracts of *Pteris cretica* L. and BHT exhibited 63.1%, 64.53% 67.97% and 89% antioxidant activity, respectively.

DISCUSSION

Among different secondary metabolites, flavonoids, tannins, saponins and alkaloids are considered to be biologically active and mainly responsible for antimicrobial and antioxidant activities.

A positive Dragendorff's test indicates the presence of alkaloids in *Pteris cretica* L. The presence of alkaloids has also been reported in five other species of genus *Pteris* (24). No alkaloid has yet been isolated from *Pteris cretica* L. to date, which demands further investigation. Five flavone glycosides have already been isolated from *Pteris cretica* L. (25-27), justifying positive flavonoid detection tests presented in Table 1. The presence of terpenoides including kaurane type of diterpenoides and some of their glycoside derivatives have also been reported, which is confirmation of positive qualitative tests for terpenoides (28).

The antibacterial activity of n-hexane extract might be due to the presence of saponins. Non-polar extracts of *Pteris multifida* and *Pteris quadriaurita* have been reported inactive against selected Gram positive and Gram negative bacterial strains (29, 30).

Partially polar (e.g., chloroform) and polar (e.g., ethanol) extracts of *Pteris vitata* and *Pteris quadriaurita* have been investigated against few Gram positive and Gram negative bacterial strains and found to be active (31).

The antifungal results correlate with the work already reported by Dalli (32).

The present research showed that *Pteris cretica* L. extracts possess antimicrobial activity against selected bacterial and fungal species. The work is also the verification of ethno-pharmacological uses of *Pteris cretica* L. as antimicrobial in the form of fronds paste on wound healing (7).

In aqueous/methanol and ethanol solution, DPPH is present as stable free radical. It has ability to accept an electron/hydrogen radical released by antioxidant to form α,α-diphenyl-β-picryl hydrazine, which is a stable diamagnetic molecule. So, it can be used to evaluate the antioxidant potential of antioxidants (33). The scavenging free radical activity of *Pteris cretica* extracts might be due to the hydrogen donating ability as already reported by Kim (34).

The mechanism of discoloration or bleaching of β-carotene can be explained by free radical mediated phenomenon resulting from the hydroperoxides formed from linoleic acid. In the absence of *Pteris cretica* L., β-carotene undergoes rapid discoloration due to the attack of linoleate free radicals and other free radicals formed in the system. The linoleate free radicals formed due to the release of hydrogen atoms from linoleic acid attack the highly unsaturated part of β-carotene molecules and ultimately double bonds are lost by oxidation. This will result in the loss of chromophore part and characteristic orange color. But in the presence of antioxidants (present in plant extracts), there is significant decrease in the oxidation process to unsaturated part of β-carotene molecules due to the neutralization of linoleate free radicals and other free radicals (35). This neutralization can be justified by retaining the color in the reaction mixture. It has already been reported that phenolic compounds with significant antioxidant activity are present in *Pteris ensiformis* (36).

CONCLUSION

The extracts of *Pteris cretica* L. showed significant antioxidant activities as well as antimicrobial activities against *Bacillus subtilus, Staphylococcus aureus*, *Clostridium sporogenes*, *Pseudomonas aeruginosa, Klebsiella pneumoniae*, *Candida albicans* and *Aspergillus oryzae*.

Among three solvent extracts, ethanol and chloroform extracts showed wide range of activities as compared to n-hexane one.

These extracts should be used for the isolation, purification and class characterization of potent antimicrobial and antioxidant compounds.

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CHEMICAL COMPONENTS AND VARIABILITY OF THE ESSENTIAL OILS ISOLATED FROM INFLORESCENCES OF *CARDUUS* SPP.

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Keywords: *Carduus* species, essential oil, GC/MS method

Carduus is a predominantly Eurasian genus of about 90 species that belongs to the *Asteraceae* family (1). That plant contains multiple secondary metabolites: flavonoids, phenolic acids, alkaloids, sterols, triterpenes, polyacetylenes, acetylenes, sterols hydrocarbons, coumarins and lignans. (1-6) There are only a few information about essential oils in *Carduus* sp. The prominent component found in essential oils obtained from aerial parts of *C. pycnocephalus* L. was palmitic acid (39.62%). In GC/MS analysis of the petroleum ether extract of the aerial parts of the plant sixteen compounds were identified. Olean-12-en-3-α-ol (20.39%), ursa-9(11),12-dien-3-ol (17.74%) and palmitic acid (17.62%) were the major components (7). Another authors identified 29 components in the oil of *C. pycnocephalus* representing 83.4% of the total oil with hexadecanoic acid (23.3%) as a main constituent (8).

The major essential oil components from flowers of *C. candicans* ssp. *globifer* were benzaldehyde (22.1%), palmitic acid (8.9%), methyl salicylate (7%), heptacosane (6%), tricosane (6.1%), pentacosane (5%), Z-12-pentacosene (3%) and β-caryophyllene (3%).

Palmitic acid (17.9%) together with methyl salicylate (14%), benzaldehyde (13.2%), trans-nerolidol (4%), p-cymen-8-ol (4%) and tricosane (2%) were detected as most characteristic compounds occurring in the essential oils from *C. thoermeri* (9). Essential oils received from aerial parts of *C. nutans* growing in Italy, were investigated and the major component of this essential oils was hexadecanoic acid (18.6%), hexahydrofarnesylacetone (7.8%), heptacosane (5.9%), 4-vinyl guaiacol (5.8%), pentacosane (3.8%) and eugenol (3.6%). (1). The aim of these studies was qualitative analysis of essential oils hydrodistilled from the inflorescences of three *Carduus* species growing in Poland. These were: *Carduus crispus* L., *C. defloratus* L. and *C. nigrescens* Vill. Essential oils from the inflorescences of investigated species have not been examined so far.

MATERIALS AND METHODS

Plant material

The inflorescence of *Carduus* L. species were collected in the Medicinal Plant Garden, of the Department of Pharmacognosy, Lublin, Poland. The inflorescence were dried in air at room temperature and powdered according to the accepted normal procedures. The procedure of preparation followed the conditions described in the Polish Pharmacopoeia VI.

Voucher specimens were given to the plants and the samples were deposited in the herbarium of The Department of Pharmacognosy, Medical University, Lublin.

Hydrodistillation

Air dried and powdered inflorescence of *Carduus* L. spp. (10.0 g) were distilled in glass Deryng's apparatus for 3 h to obtain yellowish oils according to the parameters presented in the Polish Pharmacopoeia VI (10). In this method, three-hour hydrodistilations of the plants material with water vapor with the addition of m-xylene is applied.

The above methods allowed to obtain samples of essential oils from *Carduus* spp. The samples were

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Table 1. The composition of the examined essential oils from inflorescences Carduus crispus L., Carduus defloratus L., Carduus nigrescens Vill. (hydrodistillation with m-xylene) determined by GC/MS.

Compounds name	RI* RI	(Ref. no.)	*Carduus spp.*		
			C. crispus	*C. defloratus*	*C. nigrescens*
			Peak area (%)		
2-Pentyl-furan	994	992 (16)	0.2	0.1	-
Linalool	1103	1098 (13)	-	0.3	0.5
Nonanal	1108	1103 (15)	0.9	0.2	0.4
Terpinen-4-ol	1181		-	0.1	-
p-Cymen-8-ol	1187	1183 (13)	0.9	-	0.3
α-Terpineol	1197	1195 (15)	-	0.1	-
Methyl salicylate	1201	1190 (12)	0.7	0.5	2.3
n-Decanal	1210	1203 (15)	0.6	-	0.1
Thymol methyl ether	1249		-	-	-
Thymol	1301	1290 (12)	6.8	3.4	0.3
Carvacrol	1313	1298 (12)-	0.3	0.3	
Eugenol	1366	1356 (12)	-	-	0.6
(E)-ß-Damascenone	1389	1385 (13)	0.1	0.1	0.1
β-cubebene	1392		0.0	0.1	-
Tetradecane	1400		0.2	0.1	0.1
ß-Caryophyllene	1421	1418 (12)	1.3	2.8	-
α-Humulene	1456	1454 (12)	0.1	0.6	-
E-ß-Farnezane	1460			0.1	0.2
E-ß-Ionone	1489	1483 (15)	0.2	-	0.1
ß-Selinen	1490		-	0.2	-
Tridecanal	1514	1511 (16)	0.4	0.1	0.3
(E)-Nerolidiol	1568	1567 (15)		-	0.1 0.1
Dodecanoic acid	1592	1580 (16)	-	0.3	6.4
Hexadecane	1600	1600	0.2	0.1	0.2
Humulene epoxide	1613		-	0.5	-
Tetradecanal	1616	1611 (12)	0.2	0.1	-
α- Cadinol	1658		-	0.2	-
α-Bisabolol	1684		-	-	0.5
Unknown	1711		-	-	2.7

Table 1. Cont.

Compounds name	RI* RI	(Ref. no.)	*Carduus spp.*		
			C. crispus	*C. defloratus*	*C. nigrescens*
			Peak area (%)		
Pentadecanal	1712	1711 (16)	0.9	0.4	0.3
Tetradecanoid acid methyl ester	1723		-	0.1	0.2
Unknown 1732	1732			0.4	5.1
Tetradecanoic acid	1775	1780 (16)	2.7	6.7	9.4
Octadecane	1800		-	0	0.1
Hexadecanal	1820	1815 (13)	0.3	0.1	0.1
Pentadecanone	1848		2.0	0.4	1.4
Nonadecane	1900		-	0.1	0.1
Heptadecan-2-one	1905	1900 (16)	-	0.1	0.1
Heptadecanal	1923	NIST	-	0.1	0.2
Palmitic acid methyl ester	1930	1925 (13)	1.1	0.5	0.7
Z-11-Hexadecenoic acid	1958		0.3	0.8	0.5
Palmitic acid	1992		38.7	30.9	31.9
Unknown compound 2081a	2081		0.6	0.6	0.7
Linoleic acid methyl ester	2098	2092 (14)	1.4	0.8	1.3
Linolenic acid methyl ester	2105		4.2	-	-
Docosane	2200		0.4	0.1	0.4
Tricosane	2301	2300 (15)	1.3	1.4	3.8
Tetracosane	2400		0.4	0.4	0.4
Pentacosene isomer I	2475	NIST	0.1	0.3	0.2
Pentacosene isomer II	2483	NIST	0.3	0.5	0.3
n-Pentacosane	2501		2.7	3.3	2.5
n-Hexacosane	2601		0.4	0.4	0.3
n-Heptacosane	2700		5.7	6.7	3.6
n-Octadecane	2800		0.5	0.5	0.2
Squalene	2833	NIST	0.2	0.2	0.1
n-Nonacosane	2900		2.6	2.9	0,8
Tricosane	3000		0.1	0.1	-
Hentriacontane	3100		0.1	0.3	-

RI - retention indices, * retention indices from the literature data. NIST - National Institute of Standards and Technology, Gaithersburg, MD, USA.

placed in small glass vials, dried over anhydrous sodium sulfate, and stored at 4˚C until further analysis.

Gas chromatography-mass spectrometry analysis

GC-MS method was employed for the analysis of obtained essential oils. Analysis was performed by use of Thermo-Finnigan (USA) GCQ GC-MS apparatus, working in electron impact mode. The extracts were separated on a 20 m × 0.18 mm i.d. capillary column coated with a 0.2 μm film of RT-5 (Restek). The volume of sample injected was 1 μL and split injection was used (split ratio 1 : 50).

Helium was used as carrier gas at a flow rate of 0.5 mL/min. The analyses were carried out in the programmed mode with temperature gradient of 50-320°C at 4°C/min. The final temperature was held for 1 min. The mass spectrometer in the electron impact (EI) ionization mode (70 eV), and full-scan mass spectra were recorded in the range *m*/*z* 35-500 a.m.u. (11).

The identification of individual compounds was based on the calculated retention indices, as well as was made by the comparison of received mass spectra with those of reference compounds, and available in NIST (National Institute of Standards and Technology, Gaithersburg, MD, USA) library, MS data from the literature (12-16) and our library databases.

RESULTS AND DISCUSSION

Volatile compounds identified by GC/MS method are presented in Table 1. A gas chromatograms of the essential oils from *Carduus* spp. are presented in Figures 1-3.

As it is seen there, compounds were separated and identified from the essential oils examined. Table 1 presents in detail the results of the analysis. All described compounds were identified on the basis of the comparison of their mass spectra with

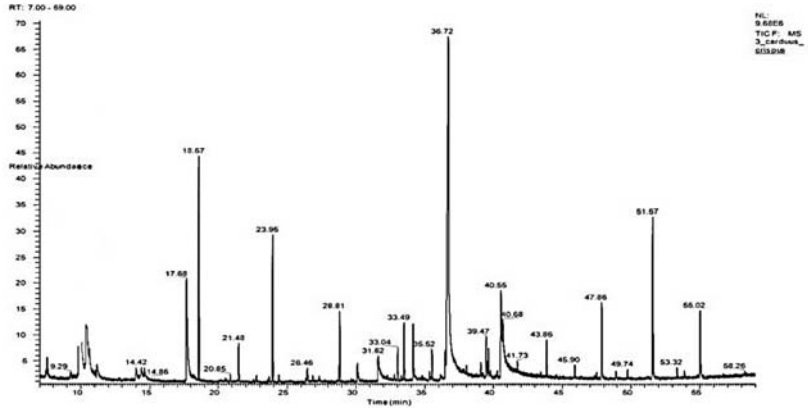

Figure 1. GC chromatogram of essentials oil (hydrodistillation with m-xylene) obtained from inflorescences of *Carduus crispus* L. The rate of temperature programming 4°C/min

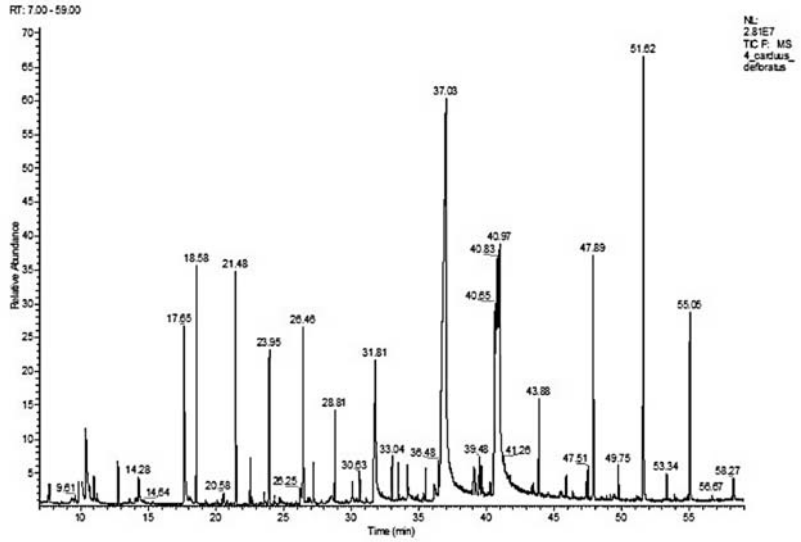

Figure 2. GC chromatogram of essentials oil (hydrodistillation with m-xylene) obtained from inflorescences of *Carduus defloratus* L. The rate of temperature programming 4°C/min

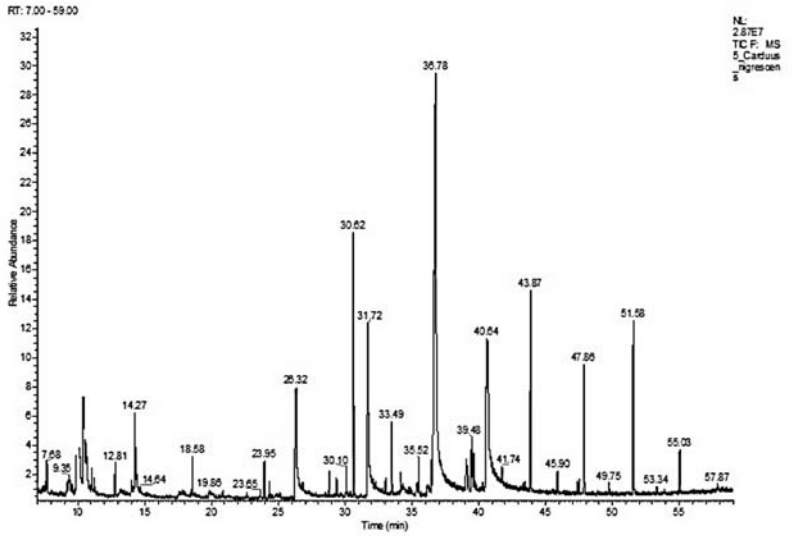

Figure 3. GC chromatogram of essentials oil (hydrodistillation with m-xylene) obtained from inflorescences of *Carduus nigrescens* Vill. The rate of temperature programming 4°C/min

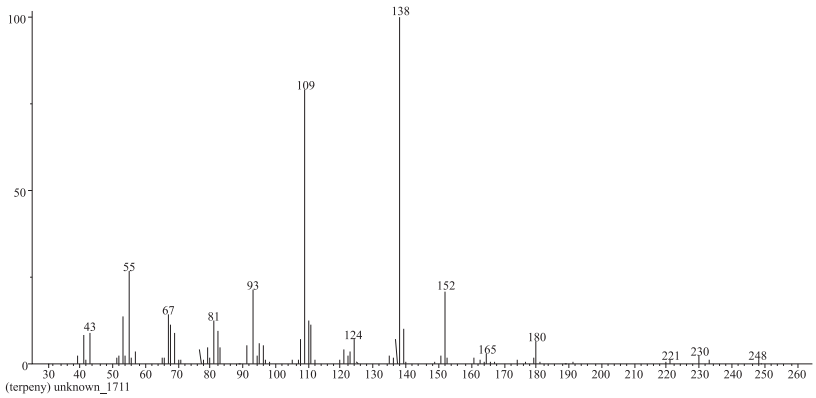

Figure 4. MS spectrum of an unknown compound with RI-1711 from essentials oil obtained from inflorescences *Carduus* L. spp.

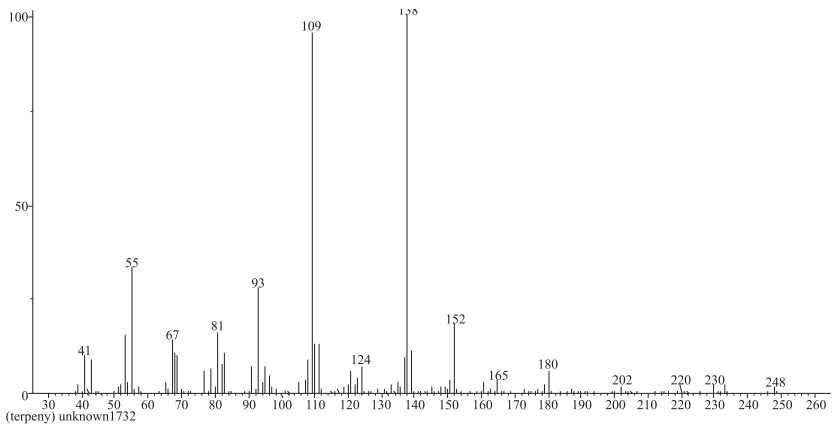

Figure 5. MS spectrum of an unknown compound with RI-1732 from essentials oil obtained from inflorescences *Carduus* L. spp.

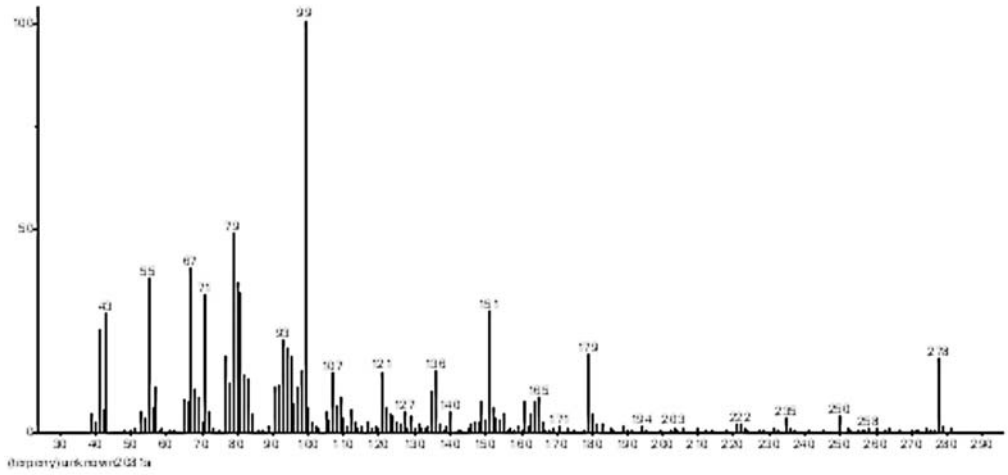

Figure 6. MS spectrum of an unknown compound with RI 2081a from essentials oil obtained from inflorescences *Carduus* L. spp.

the data available in libraries (see Materials and Methods).

The main components in volatile oil obtained from inflorescence from investigated plants are fatty acid - palmitic acids in *C. crispus* (38.7%), *C. nigrescens* (31.9%), *C. defloratus* (30.9%). Another fatty acids with odd number of carbon (linoleic myristic, lauric, linolenic and their derivatives) were also observed. Volatile oils contains aliphatic hydrocarbons with a long carbon side chain. Small amounts of terpenes were also present. Thymol is represented in *C. crispus* (6.8%), *C. defloratus* (3.4%), *C. nigrescens* (0.3%).

Carvacrol was identified in *C. crispus* and *C. defloratus* (0.3%). Eugenol was represented only in *C. nigrescens* (0.6%). Linalool was present in inflorescence of *C. defloratus* (0.3%), and *C. nigrescens* (0.5%). β-Caryophyllene is represented in *C. crispus* (1.3%) and in *C. defloratus* (2.8%). Small amount of α-bisabolol was identified only in *C. nigrescens* (0.5%). In essential oil of *C. crispus* 38 compounds were analyzed: palmitic acid (38.7%), thymol (6.8%), n-heptacosane (5.7%), linolenic acid metyl ester (4.2%),myristic acid (2.7%), n-pentacosane (2.7%), n-nonacosane (2.6%), linoleic acid (1.4%). In essential oil of *C. defloratus* 49 compounds were found: palmitic acid (30.9%), myristic acid (6.7%), n- heptacosane (6.7%), thymol (3.4%), n-pentacosane (3.3%), β-caryophyllene (2.8%), n- nonacosane (2.9%). In essential oil of *C. nigrescens* 44 compounds were analyzed: methyl salicylate (2.3%), myristic acid (9.4%), palmitic acid (31.9%), lauric acid (6.45%), tricosane (3.8%), n-heptacosane (3.6%), n-pentacosane (2.5%).

CONCLUSION

The aim of this studies were phytochemical investigations of essential oils from *Carduus* spp. inflorescence by GC–MS method. Compounds of essential oils obtained from inflorescence *Carduus* species have been identified for the first time.

Essential oils were obtained by hydrodistillation in the Deryng's apparatus. All identified compounds were identified on the basis of the comparison of their mass spectra with the data available in libraries. Most of them are ketones and aldehydes with a long carbon side chain.

Our study shows the differences in chemical composition of volatile oils obtained from different *Carduus* spp. The major component of the volatile oil received from the investigated inflorescence was palmitic acid. The percentage of this compound in the essential oils of *C. crispus* was 38.7%, for *C. nigrescens* 31.9%, and for *C. defloratus* 30.9%. Another fatty acids with odd number of carbon (linoleic, myristic, lauric, linolenic and their derivatives) were also observed. In the investigated essential oils the presence of small quantities of terpenoids were also detected These were: thymol, linalool, carvacrol, and β-caryophyllene, among others. In *C. nigrescens* α-bisabolol was identified (0.5%).

Three further unknown compounds with retention indices 1711, 1732, 2081a were also found.

Carduus sp. are sources of bioactive compounds such as thymol, carvacrol, β-caryophyllene and linalool.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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In the paper entitled „Synthesis and pharmacological activity of imidazo[2,1-*b*][1,3,4]thiadiazole derivatives", Vol. 73, issue No. 4, pp. 937-947 (2016), the correct authorship and respective affiliations should be read:

ARPIT KATIYAR[1,4], BASAVARAJ METIKURKI[2], SARALA PRAFULLA[1], SUJEET KUMAR[1], SATYAPRAKASH KUSHWAHA[1], DOMINIQUE SCHOLS[3], ERIK DE CLERCQ[3] and SUBHAS S. KARKI[1]

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The paper entitled: "RELATIVE BIOAVAILABILITY STUDY OF SUCCINIC ACID COCRYSTAL TABLET AND MARKETED CONVENTIONAL IMMEDIATE RELEASE TABLET FORMULATION OF CARBAMAZEPINE 200 MG IN RABBITS" authored by MAJEED ULLAH, GHULAM MURTAZA and IZHAR HUSSAIN, published in Acta Poloniae Pharmaceutica - Drug Research, Vol. 73, No. 4, pp. 1023-1027, 2016 has been withdrawn at Authors request.

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Scope of the Journal

Acta Poloniae Pharmaceutica - Drug Research publishes papers in all areas of research. Submitted original articles are published in the following sections: Reviews, Analysis, Biopharmacy, Drug Biochemistry, Drug Synthesis, Natural Drugs, Pharmaceutical Technology, Pharmacology, Immunopharmacology, General. Any paper that stimulates progress in drug research is welcomed. Both, Regular Articles as well as Short Communications and Letters to the Editor are accepted.

Preparation of the manuscript

Articles should be written in English, double-spaced. Full name (first, middle initial, last) and address of authors should follow the title written in CAPITAL LETTERS. The abstract should be followed by keywords. We suggest the following structure of paper: l) introduction, 2) experimental, 3) results, 4) discussion and conclusion.

Instructions for citation of references in the e-journal:

1. In the text, sequential numbers of citations should be in order of appearance (not alphabetically) in parentheses (...) not in brackets […].
2. In the list of references, for papers the correct order is: number of reference with dot, family name and initial(s) of author(s), colon, proper abbreviation(s) for journal (Pubmed, Web of Science, no dot neither coma after one word journal name), number of volume, number of issue (if necessary) in parantheses. first page or number of the paper, year of publication (in parentheses), dot. For books: number of reference with dot, family name and initial(s) of author(s), colon, title of chapter and/or book names and initials of editors (if any), edition number, page(s) of corresponding information (if necessary), publisher name, place and year of publication.

EXAMPLES:

1. Gadzikowska M., Grynkiewicz G.: Acta Pol. Pharm. Drug Res. 59, 149 (2002).
2. Gilbert A.M., Stack G.P., Nilakantan R., Kodah J., Tran M. et al.: Bioorg. Med. Chem. Lett. 14, 515 (2004).
3. Roberts S.M.: Molecular Recognition: Chemical and Biochemical Problems, Royal Society of Chemistry, Cambridge 1989.
4. Salem I.I.: Clarithromycin, in Analytical Profiles of Drug Substances And Excipients. Brittain H.G. Ed., pp. 45-85, Academic Press, San Diego 1996.
5. Homan R.W., Rosenberg H.C.: The Treatment of Epilepsy, Principles and Practices. p. 932 , Lea & Febiger, Philadelphia 1993.
6. Balderssarini R.J.: in The Pharmacological Basis of Therapeutics, 8th edn., Goodman L., Gilman A., Rall T.W., Nies A.S., Taylor P. Eds., Vol 1, p. 383, Pergamon Press, Maxwell Macmillan Publishing Corporation, New York 1985.
7. International Conference on Harmonization Guidelines, Validation of analytical procedures, Proceeding of the International Conference on Harmonisation (ICH), Commission of the European Communities, Geneva 1996.
8. http://www.nhlbi.nih.gov/health/health-topics/topics/ms/ (accessed on 03. 10. 2012).

Chemical nomenclature should follow the rules established by the International Union of Pure and Applied Chemistry, the International Union of Biochemistry and Chemical Abstracts Service. Chemical names of drugs are preferred. If generic name is employed, its chemical name or structural formula should be given at point of first citation.

Articles should be written in the Past Tense and Impersonal style. I, we, me, us etc. are to be avoided, except in the Acknowledgment section.

Editor reserves the right to make any necessary corrections to a paper prior to publication.

Tables, illustrations

Each table, figure or scheme should be on a separate page together with the relevant legend and any explanatory notes. Tables ideally should not have more than 70, and certainly not more than 140, characters to the line (counting spaces between columns 4 characters) unless absolutely unavoidable.

Good quality line drawings using black ink on plain A4 paper or A4 tracing paper should be submitted with all lettering etc., included. Good black and white photographs are also acceptable. Captions for illustrations should be collected together and presented on a separate sheet.

All tables and illustrations should be specially referred to in the text.

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The same general rules apply like for regular articles, except that an abstract is not required, and the number of figures and/or tables should not be more that two in total.

The Editors reserve the right to publish (upon agreement of Author(s) as a Short Communication a paper originally submitted as a full-length research paper.

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We encourage the use of Microsoft Word, however we will accept manuscripts prepared with other software. Compact Disc - Recordable are preferred. Write following information on the disk label: name the application software, and the version number used (e.g., Microsoft Word 2007) and specify what type of computer was used (either IBM compatible PC or Apple MacIntosh).

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