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REVIEW

THERMOSENSITIVE MICROGELS OF POLY-N-ISOPROPYLACRYLAMIDE FOR DRUG CARRIERS – PRACTICAL APPROACH TO SYNTHESIS

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Abstract: The aim of the work is to present the main actual information on the preparation of polymers, derivatives of N-isopropylacrylamide, formed into microgels. The most often used comonomers, crosslinkers, and initiator systems are gathered herein. The known methods of emulsion polymerization and precipitation polymerization are also described, including the application of the surfactants, as well as the surfactant free emulsion polymerization. Finally, the procedures of lab-scale production of microgel were evaluated in the paper, with special intact on the thermosensitive N-isopropylacrylamide derivatives for application in biomedical field.

Keywords: microgel, N-isopropylacrylamide, emulsion polymerization, precipitation polymerization

Microgels are typically defined as crosslinked polymer particles dispersed in colloidal form in a suitable medium, which usually is water. Due to the presence of specific functional groups, depending on the type of dispersion medium, they may be subject of extensive swelling (1). Sometimes, the microgels are also referred to cross-linked latex particles that swell in water, and release water as a result of changes in thermodynamic conditions, such as the presence of different additional solvents, the change in environmental temperature, pH or ionic strength of the solution. In practice, the swelling leads to significant hydration of the microgel particles. In this case, the macroscopic picture can be virtually imperceptible with the naked eye. The particles classified in a number of scientific publications as microgels vary in the terms of diameter, although they are usually in the range from 1 nm to 10 μm, some authors accept a diameter range of 50-500 nm for dried microgels. Hydrodynamic diameter of microgels is the result of osmotic pressure on the one hand. On the other hand, the elastic forces and respective tension, present in the molecule, influence the diameter.

First study, confirming receipt of the microgel, in accordance with generally accepted definition, appeared more than 70 years ago in chemical journal printed in Berlin - Staudinger and Huseman presented the synthesis and properties of polystyrene particles (2). Less than 15 years later, Baker introduced the term “microgel” for cross-linked polymer of butadiene and styrene (3). According to his observation of the resulting polymer, in contrast to previously manufactured, he found that microgels are characterized by solubility of the corresponding linear polymers in the sol state (4). In the late eighties and in nineties, microgels based on N-isopropylacrylamide (NIPA) have become extremely attractive subject of study by developing a simple and efficient method for their preparation by Pelton and Chibante (5). Schematic course of the synthesis of poly-N-isopropylacrylamide (PNIPA) is shown in Figure 1.

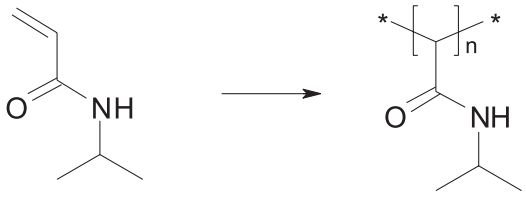

Figure 1. Scheme of PNIPA synthesis

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Poly-N-isopropylacrylamide is sometimes referred to various acronyms, such as PNIPA, PNIPAAm, PNIPAA, PNIPAm. It is a thermosensitive polymer and through the application of crosslinking agent forms a stable three-dimensional structures, known as microgels or macrogels - depending on the type of structures formed. Since the critical phase transition temperature is around 32^{o}C, in dilute solutions the expanded structure – so called coil structure - is collapsing. It transforms into a globular form when the temperature of the volume phase transition temperature is achieved, as illustrated in Figure 2. In the course of water removal from the area between the polymer chains, the loss of nearly 90% of the particles mass occurs (6). When the NIPA copolymer is applied intravenously, the elimination of circulating NIPA copolymers by the kidney is possible, if the molar mass of macromolecules does not exceed 32,000 g/mol (7).

Microgels synthesized using NIPA enjoy a growing interest among specialists in drug form technology, bioengineering and biocompatibile polymers (8-10). This is due to the above mentioned fact of removal large amounts of water from particles of PNIPA, around Volume Phase Transition Temperature (VPTT). Consequently, one can expect the release of drug substance from the microgels of PNIPA under the influence of the thermal factor. Importantly, the VPTT is in the range of known physiological temperatures, e.g., in the range of the temperature of human skin surface. By modifying the composition and structure of derivatives of NIPA it is possible to obtain a number of macromolecules with programmed VPTT in the water system. The high compatibility of this group of polymers with the tissues of the body is of extreme importance. It involves not only the chemical properties of PNIPA. The high water content in the macromolecule contributes to high biocampatibility of forming microgels. This fact enables the development of experimental methods in the field of tissue engineering, such as synthesis of resorbable implants and intervertebral discs (11, 12). In addition, the PNIPA microgels form very stable colloidal dispersions, are relatively simple to prepare, and their functionalization does not pose particular difficulties, assuming suitable manner of reaction processing. The size of the obtained particles can be well controlled, and the polydispersity index is usually maintained at a sufficiently low level. Through appropriate functionalization, i.e., through the introduction of fixed functional groups, the obtained microgels become sensitive to: the temperature factor, the factor of pH, or to changes in ionic strength of the solution. The area of potential applications of thermosensitive polymers is currently being developed intensively (13).

Initiation systems, copolymers, crosslinking agents

The first mention of the use of NIPA goes back to the fifties of the twentieth century, when it was tested due to potential repellent properties. Also the first approaches to the synthesis of NIPA polymers were done at that time. Preparation of PNIPA was carried out in different ways, although only in certain cases, suitable microgels were obtained. The straight chains of PNIPA were formed in the course of free radical polymerization. In this case, the organic solvents are used, such as methanol, benzene, tetrahydrofuran, *tert*-butanol, dioxane, and chloroform, with specific initiators: azo-bis-isobutyronitrile, benzyl peroxide or lauryl peroxide.

Another way to obtain chains of PNIPA is a process carried out using a redox initiator in aqueous

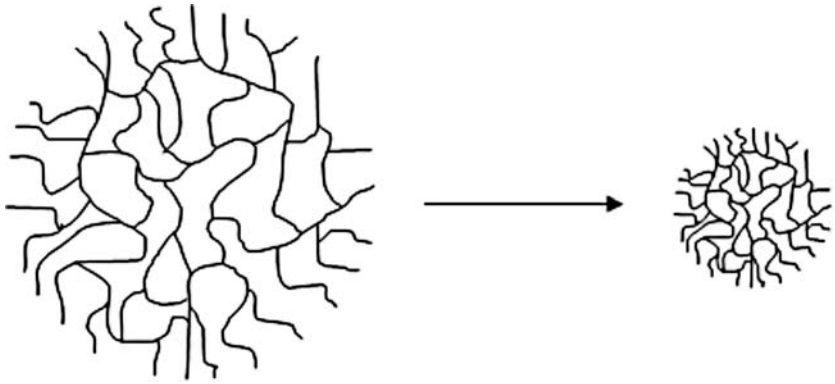

Figure 2. Depiction of volume phase transition of PNIPA microgel initiated by temperature increase in aqueous environment

Table 1. Exemplification of reactants composition in surfactant free precipitation polymerization of PNIPA.

Reactant type	Reactant	Acronyms
Initiator	Ammonium persulfate	APS
Accelerator	N,N,N',N'-tetramethylethane-1,2-diamine	TEMED, TMED
Monomer	N-isopropylacrylamide	NIPA, NIPAA, NIPAAc
Crosslinker	N,N'-methylene-bis-acrylamide	MBA, MBAm, BIS
Reaction environment	Aqueous	
Temperature	70^{o}C	

medium. The reaction is initiated using the appropriate initiator, usually: ammonium persulfate (APS), potassium persulfate (KPS) and sodium persulfate (NPS). As the initiator is sometimes also used azo-bis-isobutyronitrile (AIBN). Each of the initiator molecules, resulting in activation, may become the center of the polymerization. The spherical structures are formed around polymerization centers. They are treated in the bibliography as solid particles, dispersed in the aqueous phase. However, the resulting material is in varying degree filled with water, and connected in the areas of polymer where the hydrophilic functional groups exist. Increased temperature in the course of synthesis affects the isolation of the polymer phase with low water content from the external aqueous phase. This phenomenon manifests itself in turbidity or opalescence when the macroscopic view of the reaction mixture is observed.

The formation of hydrogels as nano- or microstructures was evaluated by numerous authors, whereas Dušek developed in details the problem of so called microsyneresis; in the case of NIPA increased temperature plays important role in microgel formation around the polymerization centers (14-16). The concentration of the initiator is also crucial for the properties of the resulting polymer. According to research of Xiao, with increasing concentration of initiator - APS in the reaction mixture, the ability of the polymer to swell increased (17). Table 1 presents the sample composition of the initial reaction mixture used to obtain microgels of PNIPA.

Free radicals derived from initiator are dissolved in water, and they remain in the solution, hence the initial reaction site is an aqueous solution, as presented in Figure 3 in stage I. As time passes, the oligomer chains with ionized groups are forming, coming from the initiator molecule. The resulting oligomers are amphiphilic, due to the presence of the hydrophilic ionic group and the lipophilic chain of the resulting polymer - Phase II. This leads over time to a micellar structures - Phase III, soon saturated with a solution of monomer (stage IV); at this point the reaction site is situated within the micelles, resulting in larger particles with time (stage V), which are stabilized by implementing an appropriate crosslinking agent. The hydrophilic groups are forming outer layer of the micelles. Due to the surface charge, the mutual repulsions stabilize the resulting colloid. Addition of electrolyte in the course of the reaction, such as sodium chloride, favors the formation of larger colloidal particles. It is caused by a reduction of repulsive forces between particles.

The main factor, used for initiation of the polymerization reaction is the temperature increase, but also visible light, ultraviolet radiation or X-ray irradiation are used (18). To ensure consistent molecular weight of macromolecules obtained in the course of the polymerization initiated in redox conditions, proper pH of the reaction mixture should be kept, using e.g., complex buffers in the pH range 6.5 or 7.4 (19). In order to accelerate the reaction it is necessary to supplement the accelerator, such as N,N,N',N'-tetramethylethane-1,2-diamine (tetramethylethylenediamine, TEMED), or sodium metabisulfite (sodium pyrosulfite). PNIPA, a linear polymer, was first comprehensively described by Howard G. Schild from Research Division of Polaroid Corporation in the early nineties of the last century (20).

In order to obtain a microgel it is necessary to ensure proper composition of the mixture of substrates, including the main monomer, comonomer, crosslinker, initiator system, and in some cases surfactant. Polymerization carried out in one reactor, the so called "batch-synthesis", requires the selection of such components, in which the individual components will react with each other with similar rate. The rate of reaction of individual comonomers affects the final composition of the resulting poly-

mer, according to the Mayo-Lewis equation (21). The compositions used for the receipt of the microgel are extremely diverse and include NIPA, the above mentioned ingredients and comonomers, e.g., acrylic acid (22), methacrylic acid and fumaric acid (23), acryl amide (24), maleic acid (25), hydroxyethyl methacrylate, N-vinylpyrrolidone (26), or N-*tert*-butylacrylamide (27, 28). The copolymers are usually used to obtain specific properties of the microgel. Introduction of anionic functional groups to the microgel can be achieved by applying suitable copolymers: unsaturated monocarboxylic or dicarboxylic acids, such as the above-mentioned acrylic acid, methacrylic acid and pentenoic acid (29). Table 2, prepared on the basis of Rzaev et al. work (30), summarizes selected comonomers used for the preparation of functionalized microgels.

Production of a stable microgel is essentially conditioned by a suitable crosslinking agent. The most commonly used crosslinking agents, and perhaps with the longest tradition of use in studies of microgels, is N,N'-methylene-bis-acrylamide (31-34). However, there are also used another crosslinkers. In previously conducted studies, special attention was paid to influence of the content of the crosslinking agent on physicochemical properties of the resulting polymer. We also evaluated the implementation of the crosslinking agent to the polymer particles (35). Among the crosslinking agents N,N'-bis-cystaminoacrylamide is applied in the synthesis (36, 37). It was observed that with increasing content of crosslinking agent increases the phase transition temperature, although the course of the hydrodynamic diameter changes as a function of increasing temperature is much milder and the phase transition point is less well visualized (38).

Between various known crosslinkers, bifunctional derivatives of polyethylene glycol are used, as factors affecting the morphology and thermosensitivity of PNIPA microgels (39). For controlled delivery of insulin, the PNIPA derivative microgels were synthesized with polyethylene glycol 400 - dimethacrylate derivative crosslinker (40, 41). Derivatives of NIPA in solution of chitosan were synthesized with the addition of ethylene glycol diacrylate which comprised four glycol units in the chain. It led to the formation of a thermosensitive gel (42). The use of ethylene glycol diacrylate with

Table 2. Choice of comonomers applied in the synthesis of copolymers of NIPA.

Group of comonomers	Examples of comonomers
Comonomers with acidic groups	Acrylic acid
	Methacrylic acid
	4-Pentenoic acid
	2-Acrylamido-2-methyl-1-propenesulfonic acid
	2-(Dimethylmaleimido)-N-ethylacrylamide
	Maleic anhydride
	Itaconic anhydride
	4-Vinylphenylboronic acid
	DNA
Acrylamide type comonomers	Acrylamide
	Acrylonitrile
	2-(Dimethylamine)propylmethacrylamide
	N-[3-(dimethylamine)propyl]methacrylamide
	2-(Dimethylmaleimido)-N-ethylacrylamide
	N-*tert*-butylacrylamide
	N-butylacrylamide
Heterocyclic comonomers	N-vinyl-2-pyrrolidone
	4-Acryloyl-morpholine
	1-Vinylimidazole
	2-Methacryloylamidohistidine
	N-acryloylpyrrolidine

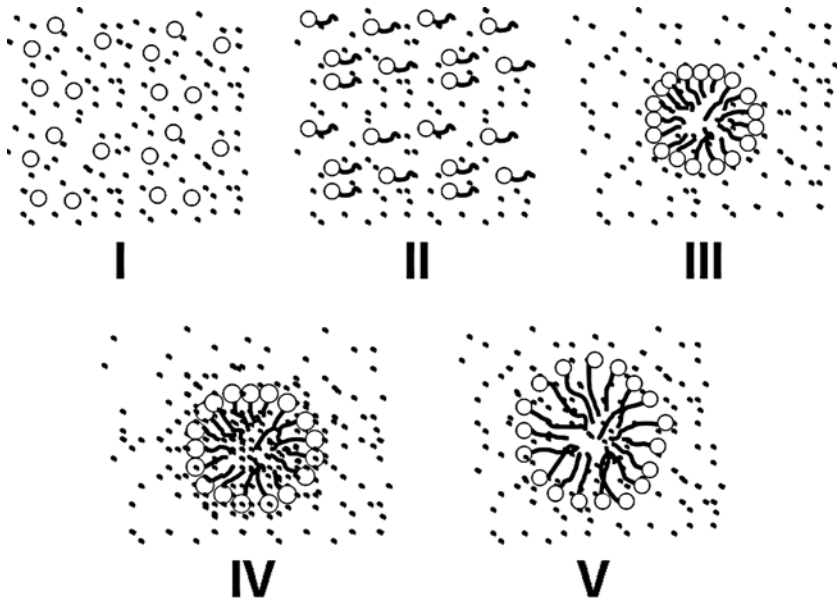

Figure 3. The changes of the locus of synthesis in the course of SFDP of NIPA derivatives, details in the text

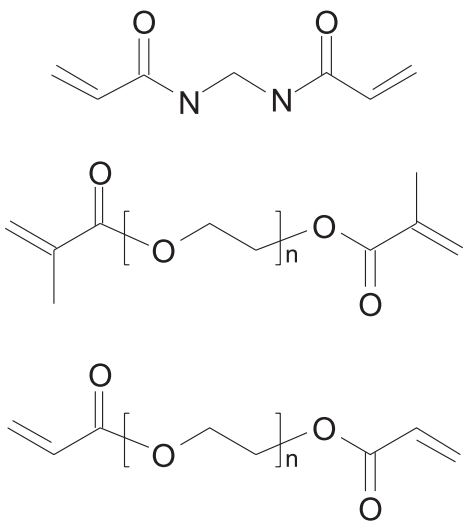

Figure 4. Crosslinking agents with varied chain length: N,N'-methylene-bis-acrylamide, glycol polyoxyethylene dimethacrylate, glycol polyoxyethylene diacrylate, respectively

one (ethylene glycol dimethacrylate, EGDMA), and three ethylene segments (triethylene glycol dimethacrylate, TEGDMA), resulted in microspheres with a diameter of 356 and 444 nm, respectively (43). In studies of crosslinking agents with different solubility in water, such as tetraethylene glycol dimethacrylate (TETGDMA), ethylene glycol dimethacrylate (EGDMA), dimethacrylate butanediol-1,3 (1,3-BDDMA), and 1,4-BDDMA the increase of solubility was accompanied by increased hydrodynamic diameter of the microgels obtained. However, the VPTT remained unchanged (44). To obtain submicron microgel some authors used glycerol dimethacrylate (GDMA), pentaerythritol triacrylate (PETA) and its propoxy derivative: pentaerythritol triacrylate propoxylate (PEPT) - in the case of crosslinking agents with the three acrylic functionals, microgels with low diameter were obtained, whereas in the case of crosslinkers with two acrylic functionals higher diameters were observed (45). Due to the possibility of handling crosslinking agents with diversified polyoxyethylene chain lengths within diacrylate and dimethacrylate derivatives, they are examined in the context of the controlled release of therapeutic substances (46). Figure 4 shows examples of crosslinking agents of different chain lengths.

Erbil et al. proposed the acrylated poly(dimethyl)siloxane (47). This resulted in a change in the VPTT, compared with NIPA polymer obtained from conventional crosslinking agent - N,N'-methylene-bis-acrylamide. More sophisticated methods include the use of biodegradable crosslinking agents, such as ACL - 3,9-divinyl-2,4,8,10-tetraoxaspiro[5.5]undekane (acid-degradable crosslinking agent) (48). Efforts are also attempted to synthesize microgel of PNIPA without the addition of crosslinking agent (49).

The course of the reaction

A key element in the course of the synthesis of microgels of PNIPA is that the NIPA is soluble in water. Along with changes in temperature also the changes in the solubility are observed, as demonstrated in studies using nuclear magnetic resonance. Concentrated solution of NIPA delaminates, at temperatures below 25°C, as the concentration excess the standard solubility of NIPA, while above 25°C the solubility is lower than the standard NIPA solubility value (50). In contrast to NIPA, its polymer is insoluble in water. However, it is true only at temperatures above the VPTT. Thus, the polymer particles are separating from the solution and in the course of polymerization the turbidity or iridescence of the reaction mixture is observed in the process of surfactant free precipitation polymerization (SFPP) or surfactant free emulsion polymerization (SFEP) (51).

Preparation of microgels is usually conducted by one of the three currently mostly available methods: by means of emulsion polymerization (EP), by precipitation polymerization (PP), and in the process of inverse emulsion polymerization (IEP). The monomer in the system remains in the form of drops, in the course of the EP reaction. In classical terms, during the EP three phases are distinguished: an aqueous phase, submicron particles of polymer, and monomer droplets. The polymerization process is supplied from the monomer droplets dispersed in the form of an emulsion in an aqueous medium, and an example of such a polymerization reaction is the formation of polystyrene. Simplified diagrams of EP and PP are shown in Figure 5A and 5B.

The aqueous phase (W) is constituted by dissolved initiator, while the monomer (M) is dispersed with the use of suitable surface active compound (surfactant-stabilized monomer droplets are shown as circles circled with dashed line). The arrow indicates the direction of migration of the monomer molecules to macromolecules of resulting polymer indicated in Figure 5A as a black dot. In the case of microemulsion polymerization, the droplets of

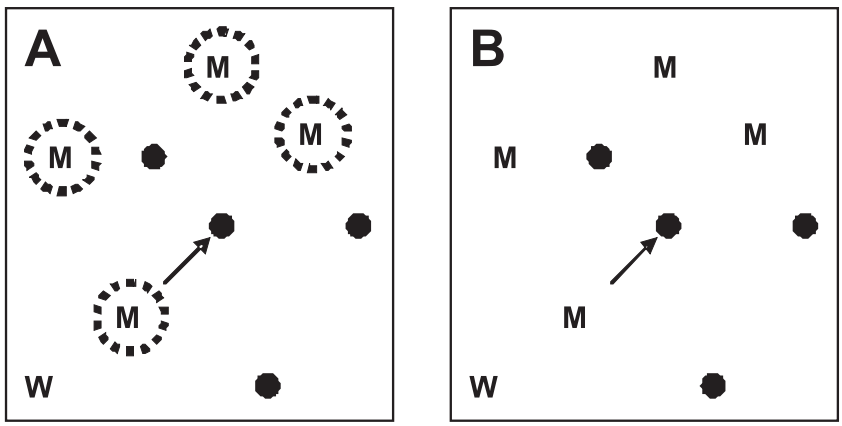

Figure 5. The scheme of EP (left panel – A) and PP (right panel – B), details in text

Table 3. Acronyms of methods evaluated for synthesis of PNIPA derivatives.

Method	Acronym
Emulsion polymerization	EP
Miniemulsion polymerization	MEP
Inverse emulsion polymerization	IEP
Surfactant free emulsion polymerization	SFEP
Precipitation polymerization	PP
Surfactant free precipitation polymerization	SFPP
Dispertion polymerization	DP
Surfactant free dispertion polymerization	SFDP

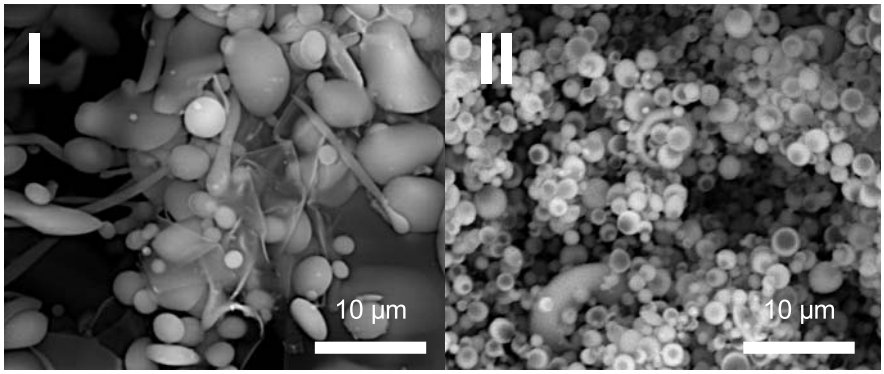

Figure 6. SEM images of NIPA derivatives, synthesized in similar conditions. The batch of microgels with higher diameter (left panel – I) was synthesized without comonomer, whereas the batch with lower diameter was synthesized with N-*tert*-butyl acrylamide as comonomer (right panel – II), details in text

monomer are characterized by very small radius of several nanometers, and this is the right place for polymerization. PP bases on the fact of precipitation of insoluble polymer obtained by polymerization of a monomer soluble in water - Figure 5B. Evaluated methods with its variations and respective acronyms are gathered in Table 3.

In the course of EP, suitable emulsifier can be used. But in some cases, the components of the reaction mixture may be maintained in the dispersed phase without adding any surfactant. In this case, we are talking about EP without surfactant i.e., SFEP. For the synthesis of PNIPA, the terms SFEP or SFPP are used in bibliography. This second definition better reflects the process, because as mentioned earlier, the monomer, NIPA, in contrast to styrene, is soluble in water and the resulting polymer is water insoluble. IEP bases on the phenomenon of polymer synthesis of monomer dissolved in the aqueous phase, which in turn is dispersed in a continuous oil phase. The advantage of this method is the possibility of incorporation of water-soluble therapeutic substances or bioactives dispersed in water, to the particles obtained in the polymerization process. Group of such methods is also referred to as the miniemulsion polymerization (MEP), although there are some differences here in relation to the IEP. Consequently, we can distinguish reversed suspension polymerization, in which monomer is suspended in the droplets of aqueous phase dispersed in the oil phase. Another way is to obtain microgels *via* microemulsion polymerization. Lin and colleagues conducted a comparison of methods for the polymerization of NIPA. According to the results of their work, they received the smallest diameter of the microgels using EP, the intermediate values were observed in the case of SFEP, while the relatively large microgels were obtained by polymerization at a temperature not exceeding 25^{o}C (52).

The use of surfactants usually affects the determined average diameter of the microgel. The diameters are in this case at a lower level than in the techniques without the use of surfactant. At the two opposite poles, in terms of size of particles obtained, are the microgels obtained by polymerization through the reverse microemulsion polymerization and through EP - in the case of the former the synthesized microgels are of 100 nm, and the monomer droplets are of diameter up to 10 nm. In the case of EP, the particle size is of about 10 µm. In the case of EP, drops can have a diameter between 10-100 microns, and in MEP the magnitude of initial monomer droplet is 30-500 nm (53). The emulsifiers used in the manufacture of microemulsions include: dodecyltrimethyl ammonium bromide and cetyltrimethyl ammonium bromide as examples of cationic emulsifiers, whereas the anionic emulsifiers are e.g.: 1,4-bis (2-ethylhexyl) sulfosuccinate sodium and sodium lauryl sulfate (54).

Emulsion polymerization without surfactant

Many authors classify methods SFPP and SFEP as polymerization by homogeneous nucleation. This means that the monomer is dissolved in a suitable solvent. As a result of the polymerization process, the polymer centers are formed, around which are growing polymer macromolecules. PNIPA, as insoluble in water, is precipitating in the course of the reaction, so the turbidity or opalescence is observed in the previously transparent system. Typically, in the reaction system all the reactants are present, except the initiator system. The introduction of the initiator initiates the sequence in which oligomers are formed, and then unstable pre-

cursor particles. Their aggregation leads to the formation of colloidally stable primary particles with unified sizes. The further course of the reaction affects the polydispersity. The polydispersity index increases as a result of aggregation of primary particles. This method may be developed to the, so called, seeding polymerization, which will play a role in synthesis in more sophisticated core polymeric structures, such as microgels with shell - core-shell microgels (55). SFEP method is successfully used for the preparation of microgels with positively charged surface - cationic microgels based on NIPA and 4-vinylpyridine (56). EP is subjected to numerous modifications, including the "semi-batch" process. In that case the reactants are added to the reaction mixture in portions - as demonstrated by Zhang et al. They obtained NIPA copolymer using the modified method, and resulting microgels were characterized by a lower diameter, while the polydispersity index was dependent on nucleation time (57). Fernandez and colleagues, in the course of polymerization of microemulsion particles, obtained PNIPA with a diameter of 30 nm using a system of benzyl peroxide initiator and TEMED (58). In Figure 6, there are images from scanning electron microscopy (SEM), of microgels from two batches of different PNIPA derivatives synthesized in the same SFEP conditions. The only difference includes the addition to the reactant mixture a lipophilic comonomer - N-*tert*-butylacrylamide. The resulting structures are smaller and more homogenous when additional lipophilic comonomer is applied (27).

Emulsion polymerization and precipitation polymerization using a surfactant

The use of an emulsifier, in concentrations exceeding the critical micellar concentration, allows the stabilization of primary particles. This mechanism enables generally to obtain particle sizes lower than in the SFEP. The surfactant is binding to the surface of primary particles and protects them against aggregation with other particles in the system. There are several interesting studies on the preparation of the polymers of NIPA by precipitation method (59). In one study, authors used nonionic surfactant Triton X-405, and in effect the microspheres were pH-sensitive and changed the structure due to the varied magnetic field – with possible application in the columns for the fractionation of DNA (60). In a similar manner the synthesis was carried out for copolymer of NIPA and acrylic acid with 2-acrylamide-deoxyglucose, in order to obtain microspheres of approximately 100 nm loaded with glucosamine (61).

Inverse emulsion polymerization

In the IEP particles are prepared from pre-gel, i.e., from droplets of a solution of monomer suitable for polymerization. The solution is dispersed in the oil phase. As a result, in the course of the reaction in aqueous phase, homogeneous microgels and aggregates of numerous microparticles smaller than the diameter of the emulsion droplets may be formed. An example of this type of polymerization is production of thermosensitive NIPA microgels, sensitive to an additional factor - the pH. Dowding et al. (62) applied here heptane as the continuous phase, while the NIPA was in the dispersed phase with MBA as a crosslinking agent. In IEP performed by Zhang et al., the microspheres were synthesized from NIPA, which enabled controlled release of ibuprofen (63). In the course of reverse MEP, the PNIPA was synthesized with the cobalt tetrafluoroborate as a soft template (64).

Dispersion polymerization

Dispersion polymerization (DP) may be considered as an interesting alternative to previously mentioned polymerization methods that affords micron-size monodisperse particles, using a single batch process. The DP is a type of PP in which the polymerization is performed from a monomer in the presence of a suitable polymeric stabilizer soluble in the reaction medium. Both the monomer and the polymeric stabilizer should be easily soluble in the applied solvent, whereas the formed polymer must be insoluble in the medium, usually an organic solvent. Initially, the system consists of a homogeneous solution of monomer with initiator and dispersant. The progression of the process leads to formation of sterically stabilized polymer particles by the precipitation of the resulting polymer. Due to the increase of monomer conversion rate, the properties of the solvent evolve. Finally, the obtained polymer particles can achieve diameter of 0.1–15 mm, and high monodispersity. Dispersant polymer may play a role as a reactive, polymerizable macromonomer. The course of the reaction may involve a block copolymer with specific affinity to the surface of the precipitated polymer, as efficient dispersant. Also application of a soluble polymer called "stabilizer precursor" with grafting feature is possible. In DP the dispersant polymer with hairy layer is a crucial factor in the process, due to the specific adsorption or incorporation onto the surface of the polymer particles obtained by PP. The type of dispersant polymer controls the stability of the colloidal system, and influences particle size of formed objects (65, 66). Lee et al. synthesized

crosslinked copolymer of NIPA and chitosan, using DP. They applied the anionic initiator APS and the cationic initiator AIBA. The homogeneous morphology was obtained in the case of APS, whereas the copolymer particles synthesized with AIBA as the initiator presented a core–shell morphology (67). Interesting structures were obtained by Akashi et al. (68), who applied as a dispersant polymer - PNIPA macromonomer 18 in ethanol, and synthesized thermosensitive microspheres 0.4–1.2 mm in diameter consisting of a polystyrene core, and PNIPA branches on the core surface. Also the magnetic microspheres of PNIPA were synthesized by the DP method; obtained particles were sensitive to the magnetic field and shrunk into an increasingly collapsed state at ca. 40^{o}C (69).

Suspension polymerization

Within the process of suspension polymerization the monomer, relatively insoluble in water, is dispersed in the form of liquid droplets, with addition of steric stabilizer. The vigorous stirring during the course of polymerization process enables production of polymer particles which are maintained in the liquid phase, however, the dispersed particles form a dispersed solid phase. The initiators must be soluble in the liquid monomer phase. Parallel terms: pearl and bead polymerization are simultaneously applied for the description of the suspension polymerization process, in the case of production of non-porous particles. The main target in suspension polymerization is to elicit uniform dispersion of monomer droplets in the liquid - aqueous phase, succeeded by precise coalescence of the droplets in the polymerization course. Several factors influence the uniformity and size of obtained polymeric forms: the interfacial tension, the agitation and the type of the reactor device. The synthesized polymeric forms usually reach the range of 10 μm to 5 mm in diameter. Application of suspending agents results in reduction of the coalescence of monomer droplets, and in the reduction of the adjacency of nascent particles. This leads to high uniformity of the dispersion of synthesized polymer. For some polar monomers, e.g., acrylic acid, the dispersing medium should be non-polar. The paraffin oils are applied in this case. The isolation of obtained particles is possible by filtration or sedimentation, especially when the beads diameter does not exceed 10 μm (70, 71). Within the process, a so called stable state is ultimately reached, in which individual drops maintain their size over prolonged periods of time. In some cases the initially low-viscous solution of liquid monomer is transformed progressively into a viscous dispersion of polymer in monomer solution; finally solid particles are observed (72).

Table 4. Choice of bibliography dealing with NIPA derivatives applied in research which aimed controlled or targeted drug delivery.

Pharmacological group	Bioactive substance	Application route	Ref.
ABA	Chlorhexidine	topical	(75)
	Lysozyme	topical	(76)
	Ofloxacin	parenteral	(77)
CVA	Fluvastatin	stent platform	(78)
	Nifedipine	topical	(79)
	Propranolol	topical	(80)
	Caffeine	topical	(81)
ACA	Doxorubicin	parenteral	(82)
	Doxorubicin	parenteral	(83)
	5-Fluorouracil	topical	(84)
	5-Fluorouracil	parenteral	(85)
LA	Procaine	topical	(86)
	Lidocaine	topical	(87)
	Bupivacaine	parenteral	(88)
NSAID's	Diclofenac	topical	(89)
	Naproxen	topical	(90)

ABA - antibacterial agents; CVA - cardiovascular agents; ACA - anticancer agents; LA - local anestetics; NSAID's - non-steroidal antiinflammatory drugs

Zhou et al. gave a detailed prescription, considering the suspension polymerization of PNIPAM microgel particles (73). The NIPAM, BIS, and sodium dodecyl sulfate (SDS) were dissolved in deionized water, heated to 70^{o}C and stirred at 200 rpm for 40 min with a nitrogen purge to remove oxygen. After nitrogen purge, the KPS dissolved in deionized water was added to initiate the polymerization. The reactant mixture was mixed at 1000 rpm for 4 h. The SDS was removed by centrifugation cycles with decantations and dispersions in deionized water, and concentrated with the use of centrifuge. Thermoresponsive macroporous poly(acrylamide-co-NIPA) microgels were synthesized by inversion suspension polymerization by Hao et al (74). The microgels possessed large surface area, which is promising factor for future applications in the area of drug delivery.

Approach to practical applications of NIPA derivatives in drug delivery

Numerous publications consider the topic of controlled or targeted drug delivery, by the use of thermosensitive polymeric carriers – nanospheres or microspheres synthesized from the main monomer NIPA, and respective comonomers. In Table 4, we present a choice of bibliography dealing with NIPA derivatives synthesized in the form of microgel, applied in research which aimed controlled or targeted drug delivery. The reviewed bibliography covers choice of original papers published in last two years in the considered area.

Due to the data presented in Table 4, the NIPA derivatives were synthesized to obtain parenteral or topical carriers for numerous drug moieties: antibacterial agents (ABA), cardiovascular agents (CVA), anticancer agents (ACA), local anestetics (LA), non-steroidal antiinflammatory drugs (NSAID). The simple and efficient method of surfactant-free DP was applied for synthesis of microgels loaded by chlorhexidine for topical drug delivery, where the temperature change was the factor influencing drug release (75). Similarly, topical application of lysozyme was proposed with the use of copolymer of carboxymethylcellulose (CMC) and NIPA – the synthesis based on decorating the backbone of CMC with linear chains of NIPA (76). Another antibacterial agent, ofloxacine, was incorported into NIPA crosslinked by acrylate terminated poly(L-lactic acid)-b-poly(ethylene glycol)-poly(L-lactic acid) (77).

In several cases CVA were loaded to the systems which consisted of NIPA derivatives. The process of DP was used to obtain matrix/microgel copolymer system for application of fluvastatin in the form of stent platform (78). Nifedipine, potent inhibitor of calcium channels was introduced into poly(2-acrylamido-2-methylpropanesulfonic acid) synthesized by single-spinneret electrospinning technique (79). Propranolol hydrochloride was combined with polyampholite N-isopropylacrylamide-based hydrogels copolymerized with acrylic acid and N-(3-aminopropyl)methacrylamide; the process of polymerization was performed *via* free radical polymerization using NIPA and cross-linker BIS in dimethyl sulfoxide (80) Caffeine, an agent applied both for cardiovascular system, as well as topical agent for skin imperfections was loaded to semitelechelic poly(*tert*-butyl methacrylate)-b-PNIPAAm brush-like polymers – the synthetic approach included complex method with chain transfer agent (81).

One of the rising branches in the field of drug micro- and nanoforms is development of potential carriers for targeted and controlled delivery of ACA. Doxorubicin with folic acid were implemented into the PNIPA-acrylamide-allylamine coated magnetic nanoparticles, which are presumed for parenteral application (82). In another approach, doxorubicin was loaded to thermo and pH dual responsive, polymer shell coated, magnetic mesoporous silica nanoparticles, obtained *via* PP (83). Nanogel of PNIPAM-co-chlorophyllin was synthesized by SFEP, and applied for the development of topically applied 5-fluorouracil (84). The targeted delivery of 5-fluorouracil was also studied in folate-targeted poly[(p-nitrophenyl acrylate)-co-(NIPA)] nanohydrogel, polymerized in the process of PP (85).

There is some information on the use of microgels synthesized *via* SFEP or PP for the controlled topical delivery of LA – procaine (86) and lidocaine (87). One study involved complex problem of parenteral application of acrylic acid-functionalized microgels loaded by bupivacaine (88). Behind the LA also the NSAID's are studied for topical applications. Diclofenac diethyl ammonium was embedded into system, which based on sodium methacrylate and NIPA, as hydrophilic/pH-sensitive and thermo-responsive monomers, and on methacrylate bovine serum albumin as cross-linker (89). Naproxen sodium release was studied from 4-vinylpyridine-based smart nanoparticles synthesized with NIPA, 2-hydroxyethyl methacrylate, and acrylic acid, obtained in the process of PP in the presence of shell-forming monomers (90). In one study, 5-aminolevulinic acid for topical application was implemented to PNIPA-co-acrylic acid microgel; the microgel was synthesized *via* microemulsion polymerization in oil-in-water system (91).

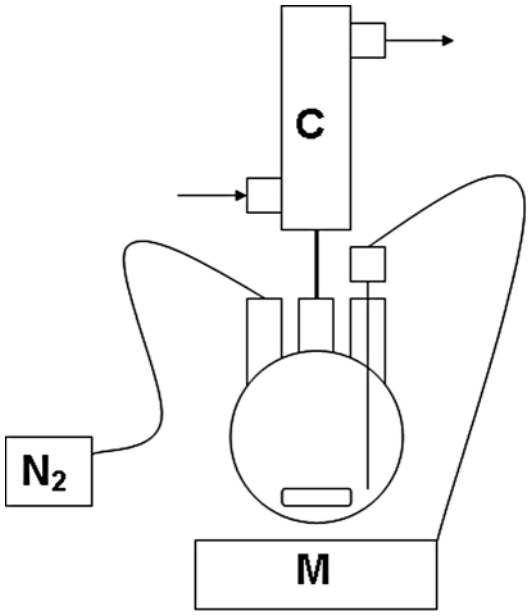

Figure 7. Synthesis set for preparation of NIPA microgels, with magnetic stirrer and feed-back control of temperature (M), cooler (C) and nitrogen inlet (N_2)

Several works dealt with the influence of newly synthesized NIPA derivatives on the release of model particles: dyes or aromatic model drugs; in some cases the dyes were introduced to reflect the structure of obtained polymer system. Interesting approach to the synthesis of drug carrier was presented by Wynter et al., who proposed microfluidic synthesis for production of copolymer in a form of monodisperse microgels, using so called co-flow glass capillary device for making single emulsion droplets of 25 – 100 μm in diameter (92). Detailed NMR study of influence of temperature on the release of five model drugs: salicylaldehyde, *m*-hydroxybenzaldehyde, ethylvanillin, 3,4-dimethoxybenzaldehyde and *p*-hydroxybenzaldehyde from beads synthesized *via* PP was described by Hofmann and Schönhoff (93). Fluorescein-labeled dextran was used in the study of magnetic composite with obtained by surfactant-free DP (94). Single fluorescein sodium salt was introduced to poly(acrylonitrile-co-NIPA) core–shell nanoparticles, obtained in a complex procedure with amidoximation and quaternization of the shell material (95). In another study, polyvinyl alcohol (PVA) matrix with nanosized pores was obtained by treatment with silica and glutaraldehyde; afterwards, the internal pores of the dry PVA matrix were filled with PNIPA, and the rhodamine B dye was used for visualization of the release process (96). Microfluidic generation of organic/aqueous/organic double emulsions and subsequent photopolymerization of the monomer residing in the aqueous phase of the droplets resulted in fabrication of PNIPA microgels containing hexadecane droplets: the system was investigated using hydrophobic dye 7-diethylamino-3,4-benzophenoxazine-2-one i.e., Nile Red, and hydrophilic dye 4',6-diamidino-2-phenylindole, to assess the characteristics of the obtained NIPA derivative system (97). Microgel beads of PNIPA-co-acrylic acid copolymer obtained by PP were studied by loading with fluorescent dye – FITC (98).

The synthesis of NIPA microgel is determined by the use or proper composition of reactants, and respective conditions of the reaction. The system usually consists of a glass reactor with a lid for the introduction of substrates to the reaction mixture and to control the course of synthesis. The controlled steps of the procedure include the fixed reaction temperature conditions, the appropriate sequence of the substrates, the isolation of the system from the influence of atmospheric oxygen, and the speed and method of stirring. Sample set for the synthesis of PNIPA microgels is shown in Figure 7.

New approaches to synthesis of NIPAM derivatives involve synthesis of cross-linked poly(N-isopropylacrylamide) microparticles in supercritical carbon dioxide (99).

SUMMARY

NIPA is a substrate for the synthesis of the corresponding copolymers with different properties and numerous possible applications in drug dosage form technology and in various branches of medicine. Synthesis of NIPA copolymers takes place mainly as a free radical process. In order to obtain microgels with the desired properties, the relevant systems, initiators and accelerators, as well as the specific comonomers and crosslinking agents must be arranged. This procedure allows one to transmit an appropriate charge to microgel surface or to maintain planned hydrophilic or lipophilic properties of the microgel. An important factor influencing the particle size obtained in the course of emulsion polymerization, is application of a surfactant. Also precipitation polymerization is often used, which therefore does not consume any surfactant. There are numerous modifications of the emulsion polymerization and they are still under development.

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ANALYSIS

DEVELOPMENT AND VALIDATION OF THE STABILITY-INDICATING LC-UV METHOD FOR DETERMINATION OF CEFOZOPRAN HYDROCHLORIDE

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Abstract: The stability-indicating LC assay method was developed and validated for quantitative determination of cefozopran hydrochloride (CZH) in the presence of degradation products formed during the forced degradation studies. An isocratic, RP-HPLC method was developed with C-18 (250 mm × 4.6 mm, 5 µm) column and 12 mM ammonium acetate-acetonitrile (92 : 8, v/v) as a mobile phase. The flow rate of the mobile phase was 1.0 mL/min. Detection wavelength was 260 nm and temperature was 30°C. Cefozopran hydrochloride as other cephalosporins was subjected to stress conditions of degradation in aqueous solutions including hydrolysis, oxidation, photolysis and thermal degradation. The developed method was validated with regard to linearity, accuracy, precision, selectivity and robustness. The method was applied successfully for identification and determination of cefozopran hydrochloride in pharmaceuticals and during kinetic studies.

Keywords: method validation, stability-indicating method, cefozopran hydrochloride

Cefozopran hydrochloride (Fig. 1) is a new, parenteral, fourth generation cephalosporin originally created by Takeda Chemical Industries. It was first registered for treatment in Japan in 1995. CZH has a broad spectrum of antibacterial activity against Gram positive such as *Staphylococcus aureus* and Gram negative bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* (1). It is stable against various β-lactamases and has low affinities to these enzymes (2). Cefozopran hydrochloride contains imidazopyridazinium methyl group at position 3, while in position 7 it is aminothiadiazomethoxyiminoacetylamino structure (Fig. 1). Those elements are responsible for broad spectrum of antibacterial activity of CZH. It is often used for antibacterial prophylaxis in abdominal surgery and for treatment of post-operative intra-abdominal infections (IAIs) (3, 4). Its recommended dose is only 2 g per day (1 g every 12 h) because of its impact on healthcare costs, and up to 4 g per day considered only for critically ill patients. In view of its good stability in solution, 1–2 g cefozopran dissolved in 100 mL saline could be infused for 1 h every 8 h (5). Its excretion rate into urine up to 24 h after administration is 82–94% and no active anti-microbial metabolite is observed in urine (6-9). CZH is well tolerated and most of adverse effects after its administration were of a mild or moderate severity, were of short period, improved spontaneously, and recovered completely (9). Since the majority of the side effects of β-lactam antibiotics are caused by their degrada-

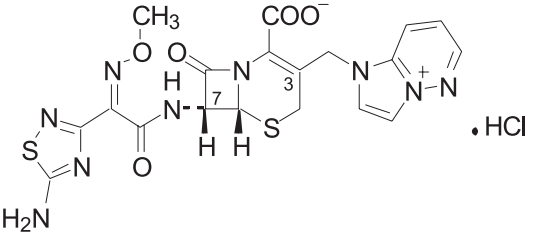

Figure 1. Chemical structure of cefozopran hydrochloride

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tion products, it is vital to improve analytical methods for the determination of β-lactam analogues. Previous studies have proved that cephalosporins are susceptible to degradation in aqueous solutions (10-17) and in solid state (18-26). Developed chromatographic method for the determination of CZH had many disadvantages like significant organic solvent consumption or incompatible to HPLC-MS water phase (27-30).

The aim of this work was to develop and validate HPLC method with UV detection suitable for identification, determination, and stability study of cefozopran hydrochloride and its degradation products.

EXPERIMENTAL

Chemicals, reagents and solutions

Cefozopran hydrochloride was obtained from CHEMOS GmbH Werner-von-Siemens Str. 3, D-93128 Regenstauf, Germany. It is white or pale yellowish white, crystalline powder soluble in water and conforms to Japanese Pharmacopeia XV standards.

All other chemicals and solvents were obtained from Merck KGaA (Germany) and were of analytical grade. High quality pure water was prepared by using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120).

Instrumentation

HPLC Dionex Ultimate 3000 analytical system consisted of a quaternary pump, an autosampler, a column oven and a diode array detector was used. As the stationary phase a Lichrospher RP-18 column, 5 µm particle size, 250 mm × 4 mm (Merck, Darmstadt, Germany) was used. The mobile phase composed of acetonitrile – 12 mM ammonium acetate (8 : 92, v/v). The flow rate of the mobile phase was 1.0 mL/min and the injection volume was 10 µL. The wavelength of the DAD detector was set at 260 nm. Separation was performed at 30°C. Photodegradation stability studies were performed using Suntest CPS$^+$ (Atlas®) with filter Solar ID65.

Procedure for forced degradation study of cefozopran hydrochloride

Stability tests were performed according to International Conference on Harmonization Guidelines (31).

Degradation in aqueous solutions

The degradation of cefozopran hydrochloride in aqueous solutions was studied in hydrochloric acid (1 mol/L) at 298 K, in sodium hydroxide (0.1 mol/L) at 298 K and in water at 373 K. Degradation was initiated by dissolving an accurately weighed 5.0 mg of cefozopran hydrochloride in 25.0 mL of the solution equilibrated to desired temperature in stoppered flasks. At specified times, samples of the reaction solutions were withdrawn and instantly cooled with a mixture of ice and water.

Oxidative degradation

Degradation was initiated by dissolving an accurately weighed 5.0 mg of cefozopran hydrochloride in 25.0 mL solution of 3% H_2O_2 equilibrated to 298 K.

Thermal degradation

Samples of cefozopran hydrochloride (5.0 mg) were weighed into glass vials. In order to achieve the degradation of cefozopran hydrochloride in solid state, their samples were immersed in heat chambers at 373 K at RH = 0%, at 373 K at RH ~ 76.4% and at 353 K at RH ~ 76.4%. At specified time intervals, determined by the rate of degradation the vials were removed, cooled to room temperature and their contents were dissolved in mixture acetonitrile and water (1 : 1, v/v). The obtained solutions were quantitatively transferred into measuring flasks and diluted with the same mixture of solvents to 25.0 mL.

UV degradation

Samples of cefozopran hydrochloride (5.0 mg) were accurately weighed, dissolved in 25.0 mL of water and then they were exposed to light according to ICHQ1b directions.

RESULTS AND DISCUSSION

It was observed that satisfactory resolution of cefozopran hydrochloride (retention time 4.6 min.) and their degradation products (retention time from 1.9 to 3.6 min.) formed under various stress conditions was achieved when analysis of stressed samples were performed on an HPLC system using a C-18 column and a mobile phase composed of 8 volumes of acetonitrile and 92 volumes of ammonium acetate, 12 mmol/L. The detection was carried out at 260 nm. The mobile phase flow rate was 1.0 mL/min. Typical retention times of cefozopran hydrochloride were about 4.6 min (Fig. 2). Peak asymmetry was 0.98.

Method validation

HPLC method was validated according to International Conference on Harmonization

Guidelines. The method was validated for parameters such as specificity, linearity, precision, accuracy and robustness.

Selectivity

The selectivity was examined for non-degraded and degraded samples (the solutions of cefozopran hydrochloride after stress conditions of hydrolysis (acid, base and neutral), photolysis, oxidation (H_2O_2) and thermal degradation.

The HPLC method for determination of cefozopran hydrochloride was found selective in the presence of degradation products as shown in Figure 2. Peaks were symmetrical, clearly separated from each other (Fig. 2).

Linearity

Linearity was evaluated in the concentration range 20-300 mg/L (10-150% of the nominal concentration of cefozopran hydrochloride during degradation studies). The samples of each solution were injected three times and each series comprised 7 experimental points.

The calibration plots were linear in the following concentration range 20-300 mg/L (n = 9, r = 0.9999). The calibration curve was described by the equation y = *ac*; y = (0.7904 ± 0.0022) *c*. The *b* value, calculated from equation y = a*c* + *b*, was not significant. Statistical analysis using Mandel's fitting test confirmed linearity of the calibration curves.

Accuracy, as recovery test

The accuracy of the method was determined by recovering cefozopran hydrochloride from the placebo. The recovery test was performed at three levels 50, 100 and 150% of the nominal concentration of cefozopran hydrochloride during degradation

Table 1. Intra-day, inter-day precision (n = 6) and recovery studies (n = 3).

Spiked concentration (mg/L)	Measured concentration ± S.D. (mg/L)	RSD (%)
Intra-day precision		
100.00 200.00 300.00	100.51 ± 0.22 199.32 ± 0.82 299.24 ± 1.14	0.15 0.39 0.36
Inter-day precision		
100.00	100.05 ± 0.54	0.51
Recovery studies		
Spiked concentration (mg/L)	Measured concentration ± S.D. (mg/L)	Recovery (%)
100.0 (~ 50%) 200.0 (~ 100%) 300.0 (~ 150%)	100.15 ± 0.65 199.25 ± 1.23 299.69 ± 1.51	100.15 99.62 99.90

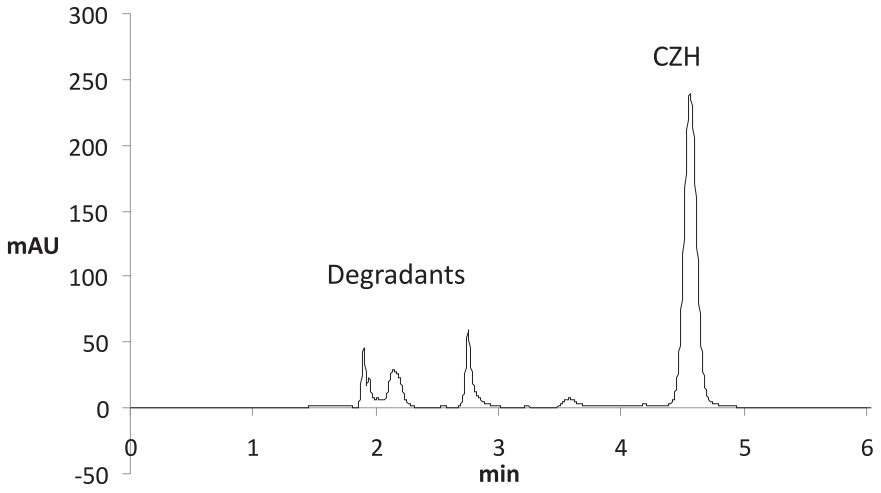

Figure 2. HPLC chromatogram of cefozopran hydrochloride (CZH) after 120 h incubation at 298 K in 1 mol/L HCl

studies. Three samples were prepared for each recovery level. The solutions were analyzed and the percentage of recoveries was calculated.

Precision

Precision of the assay was determined in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate the repeatability of the methods, six samples were determined during the same day for three concentrations of cefozopran hydrochloride. Intermediate precision was studied comparing the assays performed on two different days.

The intra-day and inter-day precision values of measured concentration of cefozopran hydrochloride, as calculated from linearity plots are given in Table 1. The RSD values were 0.15 and 0.51%, respectively, demonstrating that the method was precise.

Good recoveries were obtained for each concentration, confirming that the method was accurate (Table 1).

Limits of detection (LOD) and quantification (LOQ)

The LOD and LOQ parameters were determined from the regression equation of cefozopran hydrochloride: LOD = 3.3 S_y/a, LOQ = 10 S_y/a; where S_y is a standard error and a is the slope of the corresponding calibration curve.

Under applied chromatographic conditions, the LOD of cefozopran hydrochloride was 1.04 mg/L and LOQ of cefozopran hydrochloride was 3.15 mg/L.

Robustness

The robustness of the procedure was evaluated after changing the following parameters: the composition of the mobile phase (content of acetonitrile in the range 6–10%), the mobile phase flow rate (flow rate in the range 0.8–1.2 mL/min), wavelength of absorption (in the range 255–265 nm) and temperature (30 ± 2°C). For each parameter change, its influence on the retention time, resolution, area and asymmetry of peak was evaluated. No significant changes in resolution and shapes of peak, areas of peak and retention time were observed when above parameters were modified. Modifications of the composition of the mobile phase: organic-to-inorganic component ratio and pH resulted in the essential changes of retention time and resolution in determination of cefozopran hydrochloride.

Results of forced degradation experiments

During stability studies, degradation of 20–80% should be achieved for establishing stability-indicating nature of the assay method. In previous studies, concerning the stability of cephalosporins, it was observed that basic hydrolysis was a fast reaction (10-17). Also in the case of cefozopran hydrochloride significant degradation was observed at basic hydrolysis. Photodegradation of cefozopran hydrochloride was observed after exposition even on 1.2 million lux h (solution). It was observed that around 30% of cefozopran hydrochloride degraded under these conditions. Cefozopran hydrochloride was susceptible for degradation in solid state. At increased RH the degradation was much faster than in dry air. The results of forced degradations in various conditions are summarized in Table 2. Similar results were observed for other 4th generation cephalosporins: cefpirome sulfate (CPS) (23, 32) and cefoselis sulfate (CSS) (13, 24). CZH is more stable in solutions but easier degrades in solid state than CPS and CSS.

Table 2. Results of forced degradation studies.

Stress conditions and time studies	Degradation [%]
Acidic (1 mol/L HCl; 298 K; 77 h)	35.17
Basic (0.1 mol/ L NaOH; 298 K; 11 min)	48.76
Neutral (373 K; 65 min)	54.85
Oxidizing (3% H_2O_2; 298 K; 4 h)	23.65
Thermal (solid state; 373 K; RH~0%; 41 days)	88.68
Thermal (solid state; 373 K; RH~76.4%; 1 h)	96.83
Thermal (solid state; 353 K; RH~76.4%; 1 h)	47.61
1.2 million lux h (solution)	32.12
6.0 million lux h (solution)	83.37

CONCLUSION

The isocratic RP-LC method developed for the analysis of cefozopran hydrochloride in their pharmaceutical preparations is selective, precise and accurate. The method is useful for routine analysis due to short run time and low amounts of used solvents (acetonitrile) in mobile phase. Water phase of developed method is compatible to HPLC-MS and can be used for identification of degradation products.

Acknowledgment

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DEVELOPMENT OF CHROMATOGRAPHIC METHOD FOR DETERMINATION OF DRUGS REDUCING CHOLESTEROL LEVEL – STATINS AND EZETIMIBE

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Abstract: The presented developed HPLC method and GC method may be used to separate and determine all analyzed 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins) and ezetimibe using a single columns and a uniform methodology. In order to perform qualitative and quantitative tests of statins and ezetimibe the Symmetry C18 column 250 mm × 4.6 mm, 5 μm, the mobile phase: acetonitrile:water (70 : 30, v/v), adjusted to pH = 2.5 and a spectrophotometric detector for the HPLC method were used. For GC method column HP– 1; 30 m × 0.25 mm × 0.25 μm and FID detector were selected. All results and statistical data obtained indicate good method sensitivity and precision. The RSD values are appropriate for both newly developed methods.

Keywords: hyperlipidemia, HPLC, GC, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, atorvastatin calcium, fluvastatin sodium, rosuvastatin calcium, lovastatin, simvastatin, ezetimibe

Hyperlipidemia (HLP) is a group of lipid metabolism disturbances of various pathological background and they are characteristic in that the blood levels of cholesterol, mainly low density cholesterol (LDL) and/or triglycerides, increase. Elevated levels of total cholesterol and LDL are tightly associated with an increased risk of ischemic heart disease and disturbances in the cerebral, coronary and peripheral circulation. The most important parameter of a lipid panel includes the LDL levels – the normal levels should be below 100 mg/dL, or even below 80 mg/dL according to the current standards of the European Society of Cardiology. Moreover, the cut-off value for the HDL levels has been elevated – the normal levels are those above 40 mg/dL for men and above 50 mg/dL for women, whereas a risk factor includes levels below 40 mg/dL. The third most important parameter includes the triglyceride levels – the normal levels are the ones that do not exceed 150 mg/dL.

First-choice medicinal products used in the treatment of hyperlipidemia, mainly in order to reduce the cholesterol levels, include 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors namely statins, and aryloxyalkylcarboxylic acids derivatives, namely fibrates (1-4).

Currently, a medicinal product belonging to a new group of agents affecting the lipid metabolism in the body has been introduced – and it is called ezetimibe. Ezetimibe selectively inhibits the absorption of exogenous cholesterol from a diet and present in the bile, as well as plant sterols in the intestines. It binds to the Niemann-Pick C1 like 1 protein (NPC1L1) in the epithelium of the intestinal mucous membrane. This protein plays a vital role in the absorption of cholesterol into the cells, therefore this agent reduces the cholesterol levels in the blood plasma. However, the mechanism of action of ezetimibe is different from the one observed for agents inhibiting the intestinal cholesterol absorption, ion-exchange resins and sitosterols (plant sterols) used so far in pharmacotherapy. Moreover, it has been observed that this new medicinal product does not affect the inhibition of gastrointestinal absorption in the case of triglycerides, fatty acids, bile acids or fat-soluble vitamins (A and D vitamins). Therefore,

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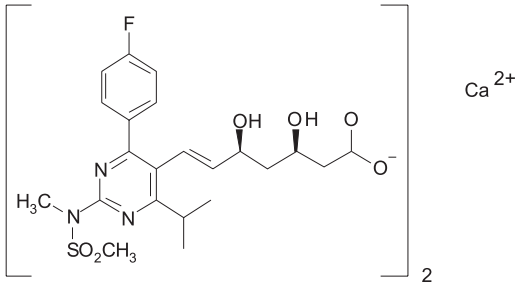
ezetimibe has been included in a new subgroup of medicinal products used in the treatment of elevated levels of total cholesterol and LDL fraction. It is used in the treatment of primary hypercholesterolemia (familial heterozygous and homozygous, and non-familial) and of familial homozygous sitosterolemia as a diet-supporting agent.

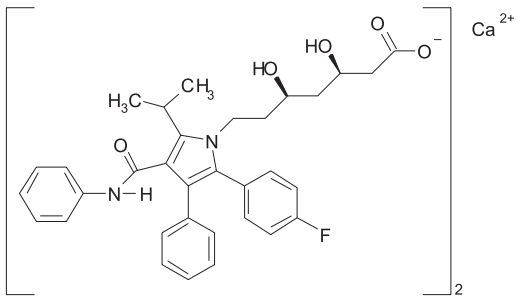
Ezetimibe is used in monotherapy or more frequently in combination therapy with statins, if the use of agents belonging to this group is contraindicated or poorly tolerated in high doses in these patients (5, 6).

Chemical formulas and names of medicinal products belonging to both therapeutic groups and used in combination therapy are presented in Figure 1.

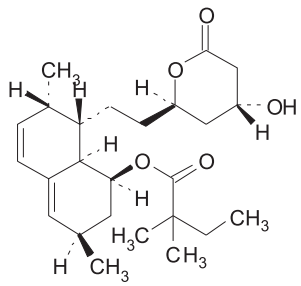
Available literature of recent years does not include any publications discussing the method to identify and determine ezetimibe in pharmaceutical



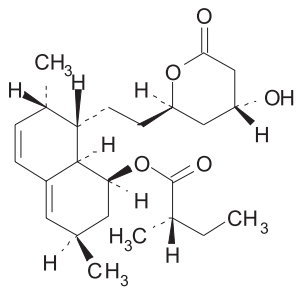
ROSUVASTATIN CALCIUM ($C_{22}H_{27}FN_3O_6S$) 2Ca
bis[(E)-7-[4-(4-fluorophenyl)-6-isopropyl-2-[methyl(methylsulfonyl)amino]pyrimidin-5-yl](3R,5S)-3,5-dihydroxyhept-6-enoic acid] calcium salt



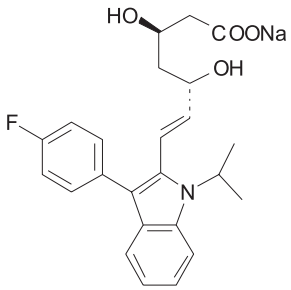
ATORVASTATIN CALCIUM ($C_{22}H_{27}FN_3O_6S$) 2Ca
[R-(R*,R*)-2-(4-fluorophenyl)-â,ä-dihydroxy-5-(1-methylethyl)-3-phenyl-4-[(phenylamino)carbonyl]-1H-pyrrolo-1-heptanoate calcium



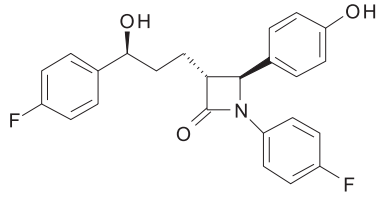
SIMVASTATIN ($C_{25}H_{38}O_5$)
(1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4R)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate



LOVASTATIN ($C_{24}H_{36}O_5$)
(1*S*,3*R*,7*S*,8*S*,8a*R*)-8-[2-[(2*R*,4R)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl]-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthtalen-1-yl (2*S*)-2-methylbutanoate



FLUVASTATIN SODIUM ($C_{24}H_{25}FNNaO_4$)
[3*R*,5*S*,6*E*]-7-[3-(p-fluorophenyl)-1-(1-isopropyl)indol-2-yl]-3,5-dihydroxy-6-heptenoate sodium



EZETIMIBE ($C_{24}H_{21}F_2NO_3$)
(3R,4S)-1-(4-fluorophenyl)-3-[(3S)-3-(4-fluorophenyl)-3-hydroxypropyl]-4-(4-hydroxyphenyl) azetidine-2-one

Figure 1. Chemical formulas and names of compounds belonging to statins and ezetimibe

products and biological materials. There are only few studies regarding clinical trials on the use of ezetimibe and statins in monotherapy and combination therapy (5, 6).

In 2006, Kublin et al. have published a study on determination of a whole group of statins using gas chromatography with the FID detector (7). In 2010, a study of a method to determine all medicinal products belonging to statins using HPLC with the Symmetry C18 column and a spectrophotometric detector was published by the same authors (8). In 2012, Kublin et al. published an article presenting test methods for the most commonly used agents belonging to statins and fibrates using HPLC and GC (9).

In modern therapy of hypercholesterolemia simvastatin or atorvastatin at a dose between 10 mg and 40 mg daily and ezetimibe at a dose 10 mg daily are used most frequently. As a result, it is possible to reduce significantly statin doses that are used and to reduce the simvastatin dose of 80 mg, which is commonly used and poorly tolerated by patients, to approx. 10–20 mg with simultaneous use of ezetimibe at a dose of 10 mg. The medicinal product called Inegy, including simvastatin and ezetimibe at a dose of 10 mg + 10 mg, has been already introduced to the American market. On the Polish market, this product has not been registered yet.

Due to lack of any reports regarding common methods for the identification and quantitative determination of agents belonging to statins and ezetimibe simultaneously, especially in medicinal products, it is justified to prepare methods to determine the contents of these compounds.

The study was assumed to prepare further simple, sensitive and unified quantification methods for the identification and quantitative determination that could be used to study a group of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (HMG-CoA), namely statins and ezetimibe in routine analyses, and to assess its analytical and economical aspects.

EXPERIMENTAL

Reference materials

Rosuvastatin calcium (MSN Laboratories Ltd.), atorvastatin calcium (Pharmathen), simvastatin (Aurobindo Pharma Ltd.), lovastatin (Teva), fluvastatin sodium (USP), ezetimibe (Merck).

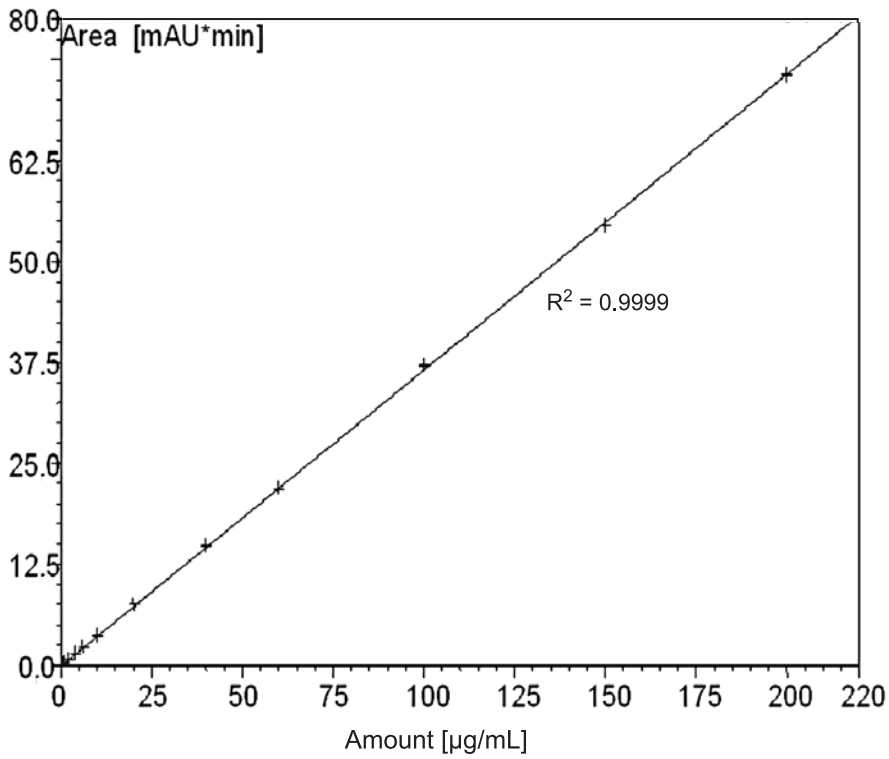


Figure 2. Regression curve for ezetimibe (HPLC)

Medicinal products

Ezetrol – tablets 10 mg (MSD-SP Limited), Simorion – coated tablets 10 mg (Orion Pharma).

Reagents and equipment

High purity HPLC reagents: acetonitrile, methanol (Rathburn), orthophosphoric acid 85% (AppliChem). Dionex liquid chromatograph with a spectrophotometric detector. Agilent Technologies gas chromatograph type 6890N with FID detector.

High performance liquid chromatography – HPLC

Qualitative analysis

Ezetimibe identification

The Symmetry C18 column, 250 × 4.6 mm, 5 µm and a spectrophotometric detector were used for the test (9, 10).

The following identification conditions were prepared: mobile phase: acetonitrile:water (70 : 30, v/v), adjusted to pH = 2.5 with 85% orthophosphoric acid; column temperature: 35°C; autosampler temperature: 15°C; mobile phase flow rate: 1.2 mL/min; injection volume: 10 µL; wavelength: 232 nm.

Preparation of calibration curve for ezetimibe

Standard solutions of ezetimibe in methanol at appropriate concentrations were prepared and a calibration curve for this compound was constructed (Fig. 2).

The linearity of the examined compound in the tested range of levels 0.010–199.92 µg/mL was observed.

The determination limit was 0.050 µg/mL, and the detection limit – 0.010 µg/mL.

Quantitative determination

Determination of ezetimibe

Under predetermined conditions, the selected ezetimibe levels were quantitatively determined in a medicinal product – Ezetrol tablets 10 mg.

A spectrophotometric detector and the wavelength of 232 nm were used during an analysis. Methanol was used as a solvent.

Preparation of standard and tested solutions

Standard solution of ezetimibe

Ca. 5 mg of a reference material - ezetimibe was weighed into a 100 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

Tested solutions

Ca. 50 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 5 mg of the active substance - ezetimibe) was weighed into a 100 mL measuring flask, approx. 50 mL of methanol was

Table 1. Retention times (R_t) of tested compounds (HPLC).

COMPOUND NAME	RETENTION TIME [min]
ROSUVASTATIN CALCIUM	2.45
EZETIMIBE	3.01
ATORVASTATIN CALCIUM	3.41
FLUVASTATIN SODIUM	3.73
LOVASTATIN	7.65
SIMVASTATIN	9.96

Table 2. Retention time (R_t) for tested compounds (GC).

COMPOUND	RETENTION TIME [min]
FLUVASTATIN SODIUM	2.26
ROSUVASTATIN CALCIUM	3.19
LOVASTATIN	4.14
SIMVASTATIN	4.41
ATORVASTATIN CALCIUM	5.51
EZETIMIBE	6.74

Table 3. Statistical assessment of results of determining the contents of ezetimibe in a medicinal product with HPLC and GC methods.

NAME OF COMPOUND / PRODUCT	NUMBER OF TESTS n	AVERAGE FROM ALL MEASUREMENTS X (%)	STANDARD DEVIATION S	CONFIDENCE INTERVAL X ± ΔX Pu = 95% (w %)	RSD (%)
Ezetimibe tablets (Ezetrol) HPLC	10	99.04	0.02	99.04 ± 0.02	0.22
Ezetimibe tablets (Ezetrol) GC	10	100.21	0.04	100.21 ± 0.04	0.55

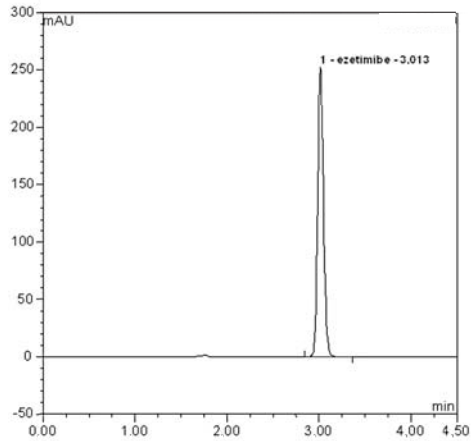


Figure 3. Chromatogram of the ezetimibe standard (HPLC)

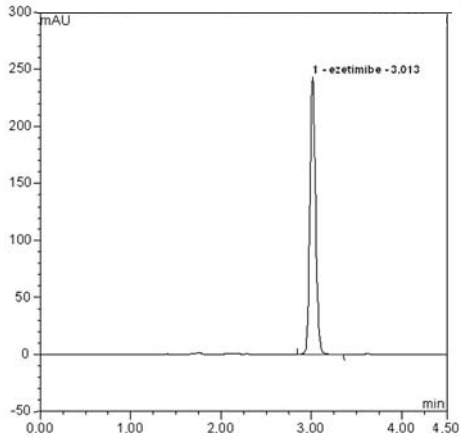


Figure 4. Chromatogram of the ezetimibe solution prepared from tablets (HPLC)

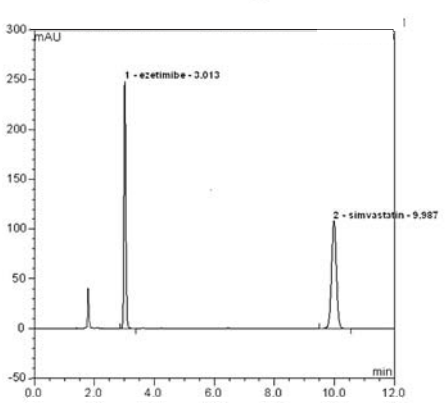


Figure 5. Chromatogram of the mixture of ezetimibe and simvastatin prepared from tablets at the wavelength of 232 nm (HPLC)

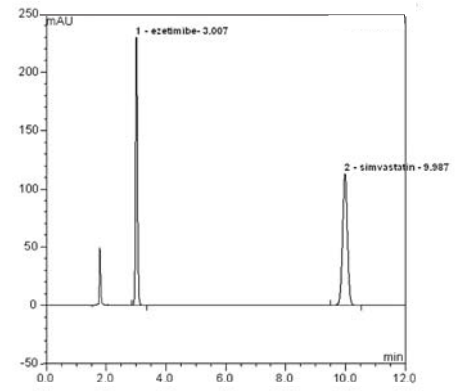


Figure 6. Chromatogram of the mixture of ezetimibe and simvastatin prepared from tablets at the wavelength of 238 nm (HPLC)

added and the solution was shaken mechanically for 30 min. Solutions were diluted to the volume, and filtered. The solutions obtained were used in tests. Table 3 presents results and a statistical assessment. Exemplary chromatograms are presented in Figures 3 and 4.

Simultaneous quantitative determination of ezetimibe and simvastatin

Medicinal products: Ezetrol and Simorion

The determination was carried out at the following wavelengths: 232 nm for ezetimibe and 238 nm for simvastatin.

Preparation of standard solution of ezetimibe and simvastatin

Ca. 5 mg of a reference material (ezetimibe) and 5 mg of a reference material (simvastatin) were transferred to a 100 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

Tested solutions

Approximately 50 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 5 mg of the active substance - ezetimibe) and 50 mg of a powdered tablet mass of Simorion (corresponding to approx. 5 mg of the active substance - simvastatin) were weighed into a 100 mL measuring flask. Approx. 50 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted with methanol and filtered. The solutions obtained were used in tests. Exemplary chromatograms are presented in Figures 5 and 6. Table 4 presents results and a statistical assessment.

Gas chromatography (GC) method

Qualitative analysis

Ezetimibe identification

The following column was used: HP– 1; 30 m × 0.25 mm × 0.25 μm and the FID detector (8, 10). The identification conditions prepared: detector temperature: 320°C; injection chamber temperature: 300°C; column temperature: programme – initial temp. 190°C for 1 min; increment of 8°C/1 min to the final temp. of 285°C/2 min; gas flow: 3.9 mL/min; injection volume: 1.0 μL and split: 10 : 1.

Preparation of a calibration curve for ezetimibe

Standard solutions of ezetimibe in methanol at appropriate concentrations were prepared and a calibration curve for this compound was drawn (Fig. 7).

The linearity in the tested range of levels 39.98–999.60 μg/mL was observed. The determina-

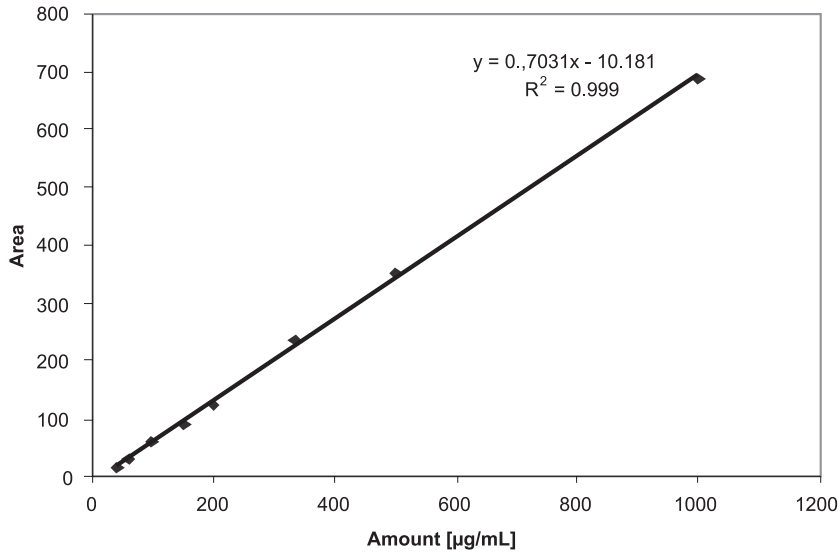


Figure 7. Regression curve for ezetimibe (GC)

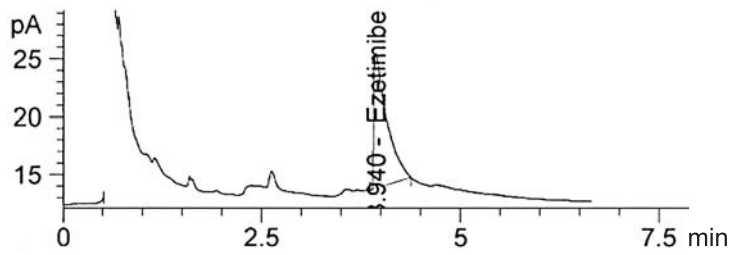


Figure 8. Chromatogram of ezetimibe standard (GC)

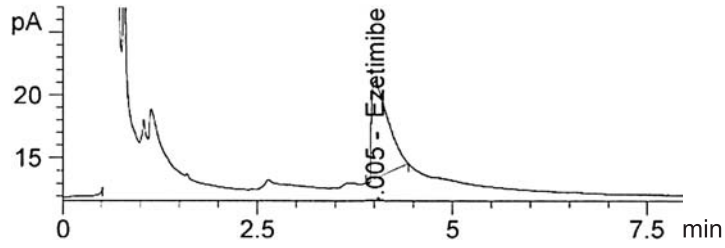


Figure 9. Chromatogram of the ezetimibe solution prepared from tablets (GC)

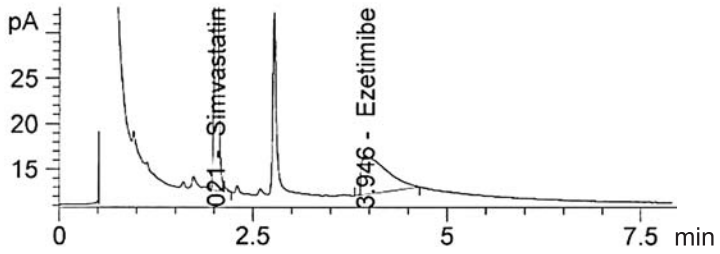


Figure 10. Chromatogram of the mixture of ezetimibe and simvastatin standards (GC)

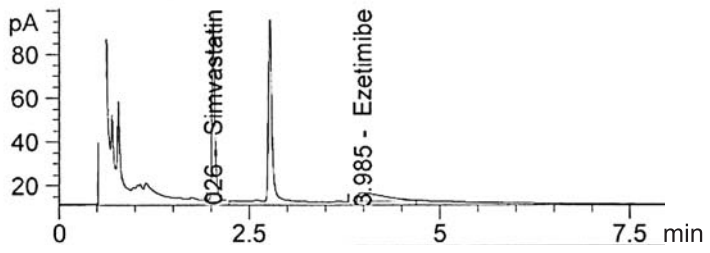


Figure 11. Chromatogram of ezetimibe and simvastatin solution prepared from tablets (GC)

Table 4. Statistical assessment of results of determining the contents of active substances in medicinal products with HPLC and GC methods parallely.

	NAME OF COMPOUND / PRODUCT	NUMBER OF TESTS n	AVERAGE FROM ALL MEASUREMENTS X (%)	STANDARD DEVIATION S	CONFIDENCE INTERVAL X ± ΔX Pu = 95% (w %)	RSD (%)
HPLC	Ezetimibe tablets (Ezetrol)	9	99.06	0.05	99.06 ± 0.04	0.46
	Simvastatin coated tablets (Simorion)	9	98.10	0.03	98.10 ± 0.02	0.27
GC	Ezetimibe tablets (Ezetrol)	**8**	99.84	0.87	99.84 ± 0.73	0.87
	Simvastatin coated tablets (Simorion)	8	95.48	0.26	95.48 ± 0.22	0.28

tion limit was 39.984 μg/mL and the detection limit – 19.992 μg/mL.

Quantitative determination

Determination of ezetimibe with the GC method

Under predetermined conditions, the selected ezetimibe concentrations were quantitatively determined in a medicinal product – Ezetrol tablets 10 mg. The FID detector was used during analyses. Methanol was used as a solvent.

Preparation of standard and tested solutions

Standard solution of ezetimibe

Ca. 5 mg of a reference material was weighed into a 25 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

Tested solutions

Ca. 100 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 10 mg of the active substance - ezetimibe) was weighed into a 50

mL measuring flask, approx. 25 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted to volume, and filtered. The solutions obtained were used in tests.

Table 3 presents results and a statistical assessment. Exemplary chromatograms are presented in Figures 8 and 9.

Parallel quantitative determination of ezetimibe and simvastatin

Medicinal products: Ezetrol and Simorion

The tests were performed under predetermined conditions used for the gas chromatography method.

Standard solution of ezetimibe and simvastatin

Ca. 5 mg of a reference material - ezetimibe and 5 mg of a reference material - simvastatin were weighed into a 25 mL measuring flask, dissolved in methanol and diluted to volume with methanol.

Tested solutions.

Ca. 100 mg of a powdered tablet mass of Ezetrol (corresponding to approx. 10 mg of the active substance - ezetimibe) and 100 mg of a powdered tablet mass of Simorion (corresponding to approx. 10 mg of the active substance – simvastatin) were weighed into a 50 mL measuring flask, approx. 25 mL of methanol was added and the solution was shaken mechanically for 30 min. Solutions were diluted to volume with methanol and filtered. The solutions obtained were used in tests. Exemplary chromatograms are presented in Figures 10 and 11. Table 4 presents results and a statistical assessment.

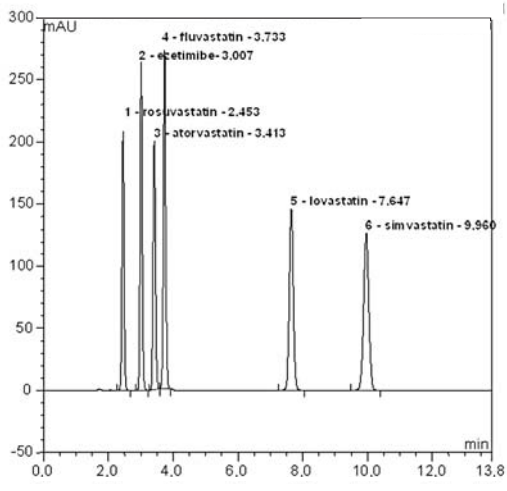


Figure 12. Chromatogram of the statin mixture and ezetimibe standards (HPLC)

Identification of the mixture containing ezetimibe and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors - statins

The parallel test to identify HMG-CoA reductase inhibitors used in pharmacotherapy – statins and ezetimibe using the HPLC method and gas chromatography method was performed.

During the HPLC method a mixture of the following substances in methanol was used: atorvastatin calcium – 52.7 μg/mL, lovastatin – 48.8 μg/mL, simvastatin – 52.2 μg/mL, fluvastatin sodium – 50.5 μg/mL rosuvastatin calcium – 51.5 μg/mL and ezetimibe – 51.9 μg/mL.

The following predetermined assay conditions were used: Symmetry C18, 250 × 4.6 mm, 5 μm column; mobile phase: acetonitrile : water (70 : 30, v/v), adjusted to pH = 2.5 with 85% orthophosphoric acid; column temperature: 35°C; autosampler temperature: 15°C; mobile phase flow: 1.2 mL/min; injection volume: 10 μL; wavelength: 232 nm.

All tested compounds were separated, and retention times obtained are presented in Table 1. The separation factor between two adjacent peaks of atorvastatin and fluvastatin (peaks 3 and 4 in Fig. 10) is 2.44. The chromatogram is presented in Figure 12.

During the gas chromatography (GC) method a mixture of following substances in methanol solutions was used: atorvastatin calcium – 52.7 μg/mL, lovastatin – 48.8 μg/mL, simvastatin – 55.2 μg/mL, fluvastatin sodium – 101.0 μg/mL, rosuvastatin calcium – 103.0 μg/mL and ezetimibe – 266.3 μg/mL

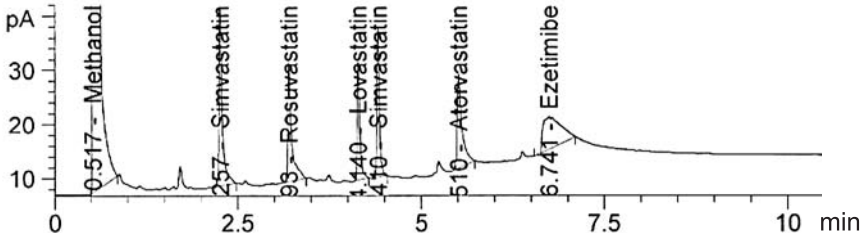


Figure 13. Chromatogram of the statin mixture and ezetimibe standards (GC)

The following identification conditions were developed: HP– 1 column; 30 m × 0.25 mm × 0.25 μm; detector temperature: 320°C; injection chamber temperature: 300°C; column temperature: programme – initial temp. 240°C for 1 min; increment of 10°C/1 min to the final temp. of 295°C, 2 min; gas flow: 3.9 mL/min; injection volume: 1.0 μL; split: 10 : 1.

All tested compounds were separated, and retention times obtained are presented in Table 2. The separation factor between two adjacent peaks of lovastatin and simvastatin (peaks 4 and 5 in Fig. 13) is 2.08. The chromatogram is presented in Figure 13.

DISCUSSION OF RESULTS

With regard to the fact that treatment of hypercholesterolemia often involves combination therapy including medicinal products belonging to statins – simvastatin the most frequently and ezetimibe, this paper was aimed to perform a quantitative analysis using HPLC and GC methods of compounds that are the most frequent ingredients of medicinal products. Results and statistical data obtained presented in Table 3 and 4 indicate good method sensitivity and precision. The RSD values presented in Table 3 and 4 are appropriate for newly developed methods to determine ezetimibe alone, as well as ezetimibe and simvastatin simultaneously.

CONCLUSIONS

HPLC and GC methods developed and presented herein may be used for simultaneous separation and determination of all tested (HMG–CoA) reductase inhibitors – statins, and a newly introduced compound – ezetimibe, using one column and similar methods.

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DRUG BIOCHEMISTRY

DIFFERENT RESPONSE OF ANTIOXIDANT DEFENSE SYSTEM TO ACAMPROSATE IN ETHANOL PREFERRING AND NON-PREFERRING RATS

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Abstract: The aim of the study was to investigate whether acamprosate, an agent attenuating relapse in human alcoholics, might modulate antioxidant status in rats chronically administered ethanol. Male Wistar rats were presented with a free choice paradigm between tap water and ethanol solution for three month to distinguish two groups of animals, preferring (PRF) and non-preferring (NPF) ethanol. Then, rats were administered acamprosate, 500 mg/kg/day, per os, for 21 days. The hepatic level of enzymatically-driven lipid peroxidation was enhanced by ethanol in PRF and NPF rats by 67 and 82%, respectively. Unstimulated microsomal lipid peroxidation was increased solely in NPF rats by 33%. Acamprosate caused 36% increase in stimulated lipid peroxidation only in NPF animals. The activities of all hepatic antioxidant enzymes examined: superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione S-transferase were decreased in rats treated with ethanol by 30 to 64% as compared to controls, however, this decrease was more distinct in ethanol preferring rats. Administration of acamprosate further reduced the activity of antioxidant enzymes only in NPF rats: catalase by 47%, glutathione peroxidase and glutathione S-transferase by 37% and glutathione reductase by 33%. No effect of acamprosate on 4-nitrophenol hydroxylase, a marker of CYP2E1 activity, was observed. As acamprosate enhanced oxidative stress only in the rats non-preferring ethanol, it could be expected that these adverse effects are not demonstrated in alcohol-dependent humans treated with acamprosate.

Keywords: ethanol, acamprosate, lipid peroxidation, antioxidant enzymes

It is generally accepted that oxidative stress plays an important role in ethanol toxicity. The ability of acute and chronic ethanol treatment to increase production of reactive oxygen species (ROS) and enhance peroxidation of lipids as well as oxidative damage of protein and DNA has been demonstrated in a variety of systems, cells and species including humans. Various pathways play a key role in ethanol-induced oxidative stress e.g., redox state changes (decrease in the NAD^+/NADH ratio) produced as a result of ethanol oxidation, effects on antioxidant enzymes, one electron oxidation of ethanol to the 1-hydroxyethyl radical. It has been shown that CYP2E1 which is specifically involved in ethanol oxidation has a high oxidase activity and plays a crucial role in the microsomal generation of ROS and of ethanol-derived free radicals (1, 2).

Acamprosate (calcium 3-acetamido-1-propanesulfate; CAS number 77337-73-6) (AC), a structural analog of ă-aminobutyric acid and homolog of taurine, has been shown to attenuate relapse in human alcoholics (3, 4). There is some evidence suggesting that AC affects glutamatergic receptor system and may act as "partial co-agonist" directly on spermidine-sensitive site of the N-methyl-D-aspartate (NMDA) receptor or *via* metabotropic receptors type 5 (mGluR5) but many aspects of its pharmacological profile are still unknown (5). Moreover, results of some research suggest that, as a taurine analog, AC can act as a ligand for taurine receptors (6). It was also noted that AC administration to laboratory animals significantly increased taurine levels in the brain (7).

Taurine has been shown to be tissue protective in many models of oxidative injury. For example,

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protective effects of taurine against hepatic steatosis and lipid peroxidation were demonstrated in rats administered ethanol (8). Taurine protects hepatocytes against H_2O_2-induced damage (9) and inhibits tert-butylhydroperoxide-induced damage to lipids in rat liver slices (10). It has been found that taurine analog, acamprosate, acts as antioxidant/free radical scavenger in the brain of rats intoxicated with alcohol (11).

The present study was undertaken to assess whether acamprosate can also prevent oxidative damage in the liver of alcoholized rats. We have used a model similar to that applied in our previous experiments, namely selectively bred alcohol-preferring and non-preferring rats (12, 13). This model has been introduced to investigate the neurobehavioral backgrounds for alcoholism and to develop efficacious therapeutic treatment (14).

The aim of the present study was to investigate whether acamprosate might modulate antioxidant status i.e., antioxidant enzymes, microsomal lipid peroxidation and GSH level in the liver of rats chronically administered ethanol.

MATERIALS AND METHODS

Acamprosate (AC) (tabl. 333 mg) was purchased from Campral, Lipha S.A., France, whereas the rest of chemicals were from Sigma Aldrich, Poland.

Experimental design

Thirty male Wistar rats (180 ± 10 g body weight) obtained from certified supplier (Laboratory Animals Breeding, Brwinów, Poland) were used in the experiment. Rats were housed individually in standard plastic cages with stainless steel covers, kept in an animal facility on reversed 12 h light-dark cycle at 20 ± 1°C, controlled humidity (65%) and circulation of air, fed Labofeed diet (ISO 9001) and tap water *ad libitum*.

The rats were presented with a free choice paradigm between tap water and ethanol solution (12% w/w) for three month with two 2-week withdrawal periods after the first and the second month. This procedure – preference development – permitted distinction of two groups of ethanol-drinking animals: (i) rats with a mean intake of ethanol about 4.9 g/kg b.w./day, preferring alcohol (PRF) and (ii) rats with a mean intake of ethanol about 1.2 g/kg b.w./day, non-preferring alcohol (NPF). Additionally, for comparative purposes, throughout the whole period of chronic ethanol treatment, an ethanol-naive control group of animals received only tap water. Both types of ethanol-drinking rats were divided into 2 subgroups, 6 rats each. In the second part of the experiment, one subgroup of PRF and one subgroup of NPF rats were treated with acamprosate (500 mg/kg b.w./day, *p.o.*, suspended in 1% methylcellulose solution) for 21 days. Two subgroups of animals, PRF and NPF, were treated *p.o.* with 1% methylcellulose solution alone for 21 consecutive days.

During the last week of drug treatment, ethanol and total fluid (sum of water and ethanol solution) intakes were measured and expressed in g/kg/day. The body weight of animals was measured after the drug treatment period.

At the end of the experiment, animals were anesthesized with ketamine (160 mg/kg b.w.; *i.p.*) and sacrificed by decapitation. The livers were removed, perfused with ice-cold 1.15% KCl and homogenized in buffered sucrose solution (Tris, pH 7.55). Microsomal and cytosol fractions were prepared by differential centrifugation according to the standard procedure. Protein concentration in the fractions was determined using Folin-Ciocalteu reagent (15). Liver homogenate for glutathione determination was prepared in phosphate buffer, pH 7.4.

The experiment was performed according to the Local Animal Ethics Committee guidelines for animal experimentation.

Biochemical assays

Microsomal lipid peroxidation in the liver was assayed in two systems: uninduced and Fe^{3+}/ADP/NADPH-stimulated (enzymatic). The level of lipid peroxidation was evaluated by measuring thiobarbituric acid reactive substances (TBARS) (16). The level of glutathione was evaluated by the determination of non-protein sulfhydryl groups concentration in liver homogenate with Ellman's reagent (17).

Antioxidant enzymes were assayed in the liver cytosol. Glutathione peroxidase (GPx) activity was determined according to Mohandas et al. (18). Hydrogen peroxide was used as a substrate. The disappearance of NADPH was a measure of the enzyme activity. Glutathione reductase (GR) was assayed by measuring NADPH oxidation using oxidized glutathione as a substrate (18). Glutathione S-transferase (GST) activity measurement was based on the spectrophotometric determination of 1-chloro-2,4-dinitrobenzene (CDNB) conjugate formed in a GSH coupled reaction (18). Superoxide dismutase (SOD) assay was based on its ability to inhibit spontaneous epinephrine oxidation (19).

Catalase (CAT) activity was determined by monitoring the rate of hydrogen peroxide decomposition (19). 4-Nitrophenol hydroxylase (PNPH) activity was determined by the method described by Reinke and Moyer (20). The method relies on the formation of 4-nitrocatechol which can be measured spectrophotometrically.

Statistical analysis

The data were expressed as the means ± SD. One way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test for multiple comparisons were used.

RESULTS

Our study showed statistically significant differences between rats in voluntary EtOH intake (Table 1). Ethanol-preferring animals (PRF) consumed significantly greater amount of EtOH than the corresponding NPF rats. Multiple AC administration resulted in a decrease in EtOH intake in PRF rats. Simultaneously, no effect of AC on EtOH intake in NPF animals was found. The differences in the total fluid intake and in the body weight between all groups were statistically insignificant.

Unstimulated lipid peroxidation was moderately increased, by 33%, only in NPF rats. Enzymatically-driven lipid peroxidation was enhanced by ethanol in PRF and NPF rats by 67% and 82%, respectively, as compared to control rats. The drug tested did not affect this parameter in PRF rats. However, in NPF group acamprosate caused 36% increase in the level of stimulated lipid peroxidation (Table 2).

The concentration of hepatic GSH was not changed in any of the groups (data not shown).

Results of antioxidant enzymes activities determination are shown in Figure 1. To facilitate the evaluation of ethanol effect on enzymes tested, the results obtained from ethanol drinking rats (PRF and NPF groups) were compared with those from control group, and PRF rats were compared with NPF rats. Effect of acamprosate was assessed by comparison of ethanol alone drinking rats with rats exposed to ethanol and acamprosate together.

SOD activity was decreased in both groups of rats administered ethanol alone, by 30% in PRF rats

Table 1. Effect of acamprosate on ethanol intake in ethanol preferring and ethanol non-preferring rats.

Treatment	Body weight (g)	Total fluid intake (g/kg/day)	Ethanol intake (g/kg/day)
Control	525 ± 44	90.7 ± 4.9	-
NPF	516 ± 39	89.9 ± 8.8	1.2 ± 0.2^{B}
NPF + AC	479 ± 34	102.1 ± 16.6	1.6 ± 1.2
PRF	476 ± 49	87.6 ± 15.2	4.9 ± 1.5^{AB}
PRF + AC	466 ± 69	104.0 ± 37.4	3.4 ± 1.5^{A}

NPF = ethanol-non-preferring rats; AC = acamprosate; PRF = ethanol-preferring rats. Results are the mean of 6 rats ± SD. Results with the same superscripts are significantly different, A = $p < 0.05$, B = $p < 0.01$.

Table 2. Microsomal lipid peroxidation in the liver of rats administered ethanol and acamprosate.

Treatment	Lipid peroxidation unstimulated (nmol/TBARS/mg protein)	Lipid peroxidation Fe^{3+}/ADP/NADPH-stimulated (nmol/TBARS/mg protein)
Control	0.48 ± 0.08^{A}	20.1 ± 3.2^{A}
PRF	0.57 ± 0.14	33.5 ± 4.9^{A}
PRF + AC	0.63 ± 0.02	40.2 ± 5.2
NPF	0.64 ± 0.08^{A}	$36.5 \pm 2.9^{A,B}$
NPF + AC	0.68 ± 0.07	49.7 ± 4.4^{B}

PRF = ethanol-preferring rats; NPF = ethanol-non-preferring rats; AC = acamprosate. Results are the mean of 6 rats ± SD. A - PRF and NPF groups are compared with control group; B - groups fed alcohol alone (PRF and NPF) are compared with rats fed alcohol + acamprosate; values with the same superscripts are significantly different, $p < 0.01$.

and by 41% in NPF rats. Acamprosate did not affect the SOD activity in ethanol drinking rats.

CAT activity was diminished by 30% in PRF rats and raised by 26% in NPF rats. Administration of acamprosate had no effect on CAT activity in PRF groups, while in NPF animals acamprosate caused the reduction of enzyme activity by 47% as compared to that in rats receiving ethanol alone.

The activity of GPx was decreased in both groups of rats receiving ethanol, in PRF rats - by 64%, while in NPF rats the decrease was small and insignificant. Administration of acamprosate to PRF rats caused a weak insignificant increase in this enzyme activity as compared to that of the rats receiving ethanol alone. Conversely, in rats with lower ethanol intake (NPF) a decrease in the GPx activity by 37% after acamprosate administration was observed.

The activity of glutathione reductase (GR) was decreased in PRF and NPF group by 51% and 30%, respectively, as compared to controls. No changes in GR activity in PRF rats administered acamprosate were found. In NPF group the activity of the enzyme was lowered by 33% after acamprosate administration.

The activity of GST in PRF group was decreased by 31% in comparison with controls. In NPF rats the decrease in GST activity was statistically insignificant. Acamprosate administration to

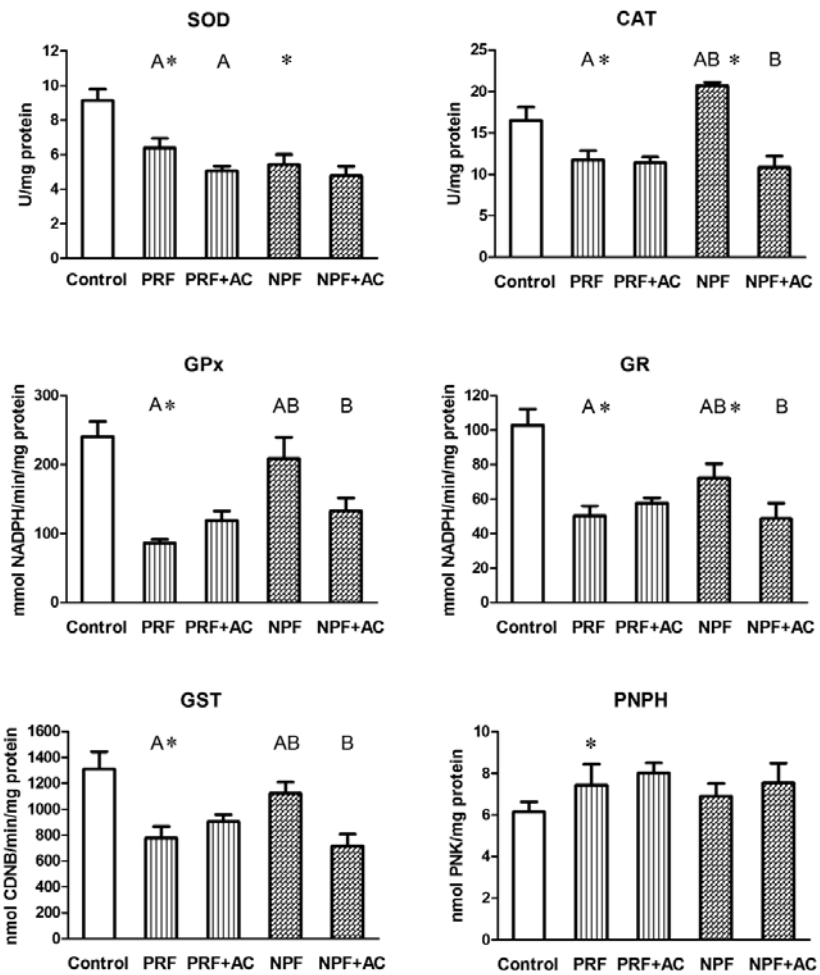

Figure 1. Activity of antioxidant enzymes and p-nitrophenol hydroxylase in the liver of rats administered ethanol and acamprosate
PRF = ethanol-preferring rats; NPF = ethanol-non-preferring rats; AC = acamprosate; SOD = superoxide dismutase; CAT = catalase; GPx = glutathione peroxidase; GR = glutathione reductase; GST = glutathione S-transferase; PNPH = p-nitrophenol hydroxylase. Results are the mean of 6 rats ± SD. * PRF and NPF groups are compared with control group (without AC treatment); A) PRF group is compared with NPF and with PRF + AC groups; B) NPF group is compared with NPF+AC group. Values with the same superscripts are significantly different, $p < 0.01$

NPF rats caused further reduction of GST activity by 37%.

4-Nitrophenol hydroxylase activity was slightly increased, by 20%, only in PRF rats receiving ethanol alone. Acamprosate did not affect this enzyme activity in rats drinking ethanol (Figure 1).

DISCUSSION

Two groups of rats (PRF and NPF) differing in voluntary ethanol intake were used in this study and the observed differences in alcohol drinking were in line with our previous report (13). The ethanol drinking pattern observed in the PRF group (> 4.0 g/kg/day) was in agreement with the suggestion, that such level of drinking may be considered as "preference" (21). The dose of AC (500 mg/kg, *p.o.*) was chosen on the basis of the previous findings (12). AC treatment led to a decrease in EtOH intake in PRF rats, what was in accordance with antialcoholic activity of acamprosate.

In the majority of chronic experiments with ethanol feeding an increase in hepatic lipid peroxidation in rodents was observed (22, 23). However, numerous studies using ethanol-feeding models failed to show the increase in lipid peroxidation (24, 25). It was demonstrated that enhanced liver lipid peroxidation was not a constant feature after long term ethanol consumption. It may be due to adaptive processes that result in an enhanced antioxidant defense (24).

In our experiment, enzymatically-driven microsomal lipid peroxidation level in the liver of rats fed ethanol alone was increased to a greater extent than non-stimulated lipid peroxidation level. The assay using enzymatic induction of lipid peroxidation aimed at assessment of the resistance of microsomes of rats fed ethanol to oxidative damage. Our results demonstrated that the liver microsomes of NPF rats exposed to the lower dose of ethanol were less resistant because the level of lipid peroxidation was higher in this group of rats. Thus, it could be suggested that in ethanol-preferring rats some adaptive mechanisms counteracting ethanol-related increase in ROS level started earlier or were more efficient than in NPF rats.

There was a difference in the TBARS level in PRF and NPF rats administered acamprosate. The increase in the level of enzymatically-driven lipid peroxidation was observed only in livers of NPF rats administered acamprosate. It should be emphasized that this increase was consistent with the reduced activity of hepatic antioxidant enzymes in the same group.

Many authors reported a decrease in GSH level in acute intoxication with ethanol (24). It is suggested that GSH depletion is caused by the enhancement of oxidative process mediated by Fe^{2+} ions liberated from ferritin and hemosiderin by ethanol. Similarly to that referring to antioxidant enzymes, the data on liver GSH in animals chronically treated with ethanol are inconsistent. In several experiments the increased GSH concentration in livers of rats fed ethanol was found (25, 26), some authors reported that GSH levels remain unchanged (27). The present studies confirmed the latter findings. According to Oh et al. (25) these discrepancies in the GSH levels might have originated from differences in the strain of rodents used and the dose as well as duration of ethanol administration.

The results of this study show that the activity of hepatic antioxidant enzymes was decreased in rats treated with ethanol alone as compared to that in control group, except for catalase which activity was raised in NPF rats. However, this decrease was greater in ethanol-preferring rats (difference insignificant only for SOD). This is consistent with the free radical theory of ethanol toxicity - the greater the dose of ethanol the greater generation of ROS and inactivation of antioxidant enzymes.

Generally, it was evidenced that an acute ethanol load elicits decreased efficiency of antioxidant enzymes (24). However, there are some controversies regarding the changes in antioxidant enzymes activity in animals chronically exposed to ethanol. It is suggested that chronic intake of ethanol induces CYP2E1 and triggers some adoptive mechanisms counteracting the impairment of cell antioxidant defense system including antioxidant enzymes (1). We have found that in PRF rats GPx activity was markedly reduced as compared to that in control group. In rats with lower intake of ethanol (NPF) the decrease in GPx activity was not statistically significant. In several experiments with a similar protocol (i.e., 4 week ethanol drinking, 6-16 g/kg b.w./day) a decrease in this enzyme activity was also found (25, 26). In two other reports the activity of GPx was not changed (28, 29). On the contrary, the increase in this enzyme activity was observed in rats receiving ethanol in a dose of 5.0 g/kg for 5 and 6 weeks (30). In our experiment GR activity was reduced to a greater extent in PRF than in NPF rats. Similar response of GR to ethanol was reported by Mallikarjuna et al. (31). However, some authors demonstrated an increase in GR activity in rats fed ethanol for several weeks (25, 26). It was hypothesized that the explanation might be the enhancement of gene expression mediated by antioxidant respon-

sive element (ARE) as a response to free radicals generated by ethanol (32). The decrease in GST activity in rats fed ethanol found in the present experiment was not confirmed by reports of other authors, who found that chronic treatment of rats with ethanol resulted in the raise in GST activity (25, 29, 31). It was suggested that the induction of GST by ethanol could be considered as an adaptive response to ethanol-induced oxidative stress. Enhanced GST activity facilitates conjugation of cytotoxic aldehydes, such as 4-hydroxynonenal, with GSH and reduction of lipid hydroperoxides, thus preventing lipid peroxidation (31).

The increase in CAT activity observed in our experiment in rats treated with the lower dose of ethanol, e.g., in NPF rats, was also reported by Oh et al. (25). It is consistent with the interpretation that in animals chronically intoxicated with ethanol some adaptive mechanisms are triggered. However, in PRF rats the activity of CAT was lower than in controls, which could be due to CAT inactivation by free radicals generated by the higher dose of ethanol. SOD activity was decreased in both PRF and NPF animals which was confirmed by other authors' findings (23, 29, 31). The changes in SOD, CAT and GPx in rats fed chronically ethanol reported in literature appear highly contradictory (24). It could be suggested that the response of antioxidant enzymes to chronic ethanol feeding depends on both, the dose and the time of exposure.

In PRF rats acamprosate did not change the activity of antioxidant enzymes. However, the activity of the majority of enzymes in NPF group was decreased after acamprosate administration. SOD was the only enzyme whose activity was not affected by acamprosate in any group of rats fed ethanol. In the available literature we have found one report concerning the effect of a acamprosate analogue, taurine, on hepatic antioxidant enzymes in rats intoxicated with ethanol. Pushpakiran et al. (33) found about 50% decrease in the activity of SOD, CAT and GPx after ethanol administration in a dose of 6 g/kg/day and the recovery of all enzymes activity to the basal level in rats fed simultaneously taurine for 28 days.

The results of the current study differ from those presented by Pushpakiran et al. (33) and Balkan et al. (34), however, direct comparison of the data obtained in our experiment and those in the reports cited is not justified because we used taurine analogue, acamprosate, not taurine itself, which might affect antioxidant enzymes in a different way. In our experiment acamprosate, a potential source of taurine (7), caused a decrease in antioxidant enzymes activity only in rats fed the lower dose of ethanol. The different response of antioxidant enzymes to acamprosate in PRF and NPF rats was apparently associated with the dose of ethanol, however, the mechanism of this relationship is not clear. It could be suggested that this difference might be due to some adaptive mechanism evoked by the long term exposure to ethanol. Probably this adaptive process appeared earlier in PRF rats consuming the higher dose of ethanol. Hence, hepatic antioxidant enzymes in this group were more resistant to changes caused by acamprosate.

Chronic ethanol consumption leads to an increase in the content of CYP2E1 in the liver and enhances its catalytic activity in the microsomal fraction. In numerous reports induction of CYP2E1 in alcohol-fed animals has been shown to increase the lipid peroxidation in hepatic microsomes (35). It is known that CYP2E1 plays an important role in the generation of hydroxyethyl radical during chronic feeding of ethanol and that ethanol-derived free radicals are major contributors to ethanol-induced oxidative stress and liver injury (1). In the current experiment, the activity of 4-nitrophenol hydroxylase (PNPH), which is known to be CYP2E1 dependent, was assayed (36). The activity of this enzyme was slightly increased only in PRF rats administered ethanol alone. In the other groups no changes in PNPH activity were observed. Similar results were reported by Kerai et al. (22), who did not observe any increase in PNPH activity in rats fed ethanol in liquid diet for 28 days. As in the current experiment, the increase in PNPH activity was noticed only in PRF rats receiving higher dose of ethanol, it could be suggested that the dose of ethanol consumed by NPF rats was too low to induce CYP2E1 activity. The lack of marked CYP2E1 induction observed in our experiment, is consistent with low level of unstimulated lipid peroxidation in the hepatic microsomes of rats chronically fed ethanol. Acamprosate did not affect the activity of CYP2E1 although it was shown that acamprosate analogue, taurine, can inactivate this isoform of cytochrome P450 (22).

Summing up, some consistency in the effects of acamprosate on the parameters of alcohol-induced oxidative stress in the liver was observed, namely, an increase in the level of lipid peroxidation and a decrease in the activity of antioxidant enzymes. However, these effects were restricted to rats non preferring alcohol. Hence, it could be expected that, by analogy to our findings, these adverse effects are not demonstrated in alcohol-dependent humans treated with acamprosate.

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IMPACT OF GENTAMICIN ON ANTIOXIDANT ENZYMES ACTIVITY IN HEMn-DP CELLS

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Abstract: Aminoglycoside antibiotics, including gentamicin, are widely used clinically in treatment of bacterial infections. Unfortunately, their side effects, especially nephrotoxicity and ototoxicity remain a problem. It is known that aminoglycoside antibiotics bind well to melanin biopolymer, but the relation between their affinity to melanin and ototoxicity is not well documented. The aim of this work was to examine the impact of gentamicin on antioxidant enzymes activity in cultured dark pigmented normal human melanocytes (HEMn-DP). The WST-1 assay was used to detect gentamicin cytotoxic effect. The analyzed antibiotic induced concentration-dependent loss in melanocytes viability. The value of EC_{50} was found to be 7.5 mM. Significant changes in the cellular antioxidant enzymes: SOD, CAT and GPx were stated in melanocytes exposed to gentamicin, what may indicate the depletion of antioxidant defense system. It is concluded, that the results obtained *in vitro* may explain a potential role of melanocytes and melanin in the causative mechanisms of aminoglycosides ototoxic effects *in vivo*.

Keywords: gentamicin; melanocytes; superoxide dismutase; catalase, glutathione peroxidase

Gentamicin is a widely used aminoglycoside antibiotic, however the clinical usefulness of this drug is limited due to its ototoxicity and nephrotoxicity. Nephrotoxicity is reversible, but destruction of auditory system is irreversible, resulting in permanent hearing loss (1-5). Many studies have reported that gentamicin-induced ototoxicity is mediated by reactive oxygen species (ROS) (1, 6, 7). Overproduction of ROS triggers the signaling pathways of cellular apoptosis, resulting in inner ear damage. Several agents that scavenge ROS or block their formation (8-10) may be considered as protective substances that reduce ROS-induced ototoxicity (11, 12).

Melanin is a unique pigmented biopolymer synthesized by melanocytes in human and various animals species. In humans, melanin is found in the skin, eyes, brain and ear (13, 14). Many investigators have demonstrated the affinity of natural and synthetic melanins for various drugs by *in vivo* and *in vitro* studies. It is generally accepted that the ability of melanin-containing tissue to accumulate and retain these drugs is remarkable (15-17).

In view of the fact that melanin is an abundant constituent of the inner ear, it seems reasonable to suspect that the specificity of gentamicin ototoxicity may result from its ability to form a complex with melanin. This phenomenon may contribute to the accumulation of the antibiotic in the inner ear and facilitate a toxic effect on surrounding cells.

Previously, we have documented that gentamicin, kanamycin, amikacin, neomycin, tobramycin and netilmicin (18-23) form stable complexes with model synthetic melanin *in vitro*. Our studies have also demonstrated that amikacin (24), kanamycin (25), netilmicin (26) and streptomycin (27) suppress melanin biosynthesis and cause depletion of antioxidant defense system in human light pigmented melanocytes (HEMa-LP).

To our knowledge, the effect of gentamicin on biochemical processes in melanocytes has never been reported. Therefore, we investigated the antioxidant enzymes activity in gentamicin-treated dark pigmented melanocytes HEMn-DP.

EXPERIMENTAL

Chemicals

Amphotericin B was purchased from Sigma-Aldrich Inc. (USA). Gentamicin and neomycin sul-

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fates were obtained from Amara, Poland. Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254 and human melanocytes growth supplement-2 (HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A. (Poland).

Cell culture

The normal human dark pigmented melanocytes (HEMn-DP, Cascade Biologics, UK) were grown according to the manufacturer's instruction. The cells were cultured in M-254 medium supplemented with HMGS-2, penicillin (100 U/mL), neomycin (10 μg/mL) and amphotericin B (0.25 μg/mL) at 37°C in 5% CO_2. All experiments were performed using cells in the passages 7-10.

Cell viability assay

The viability of melanocytes was evaluated by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay. WST-1 is a water-soluble tetrazolium salt, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO_2 for 48 h. Then, the medium was removed and cells were treated with gentamicin solutions in a concentration range from 0.01 to 10 mM. After 21 h incubation, 10 μL of WST-1 were added to 100 μL of culture medium in each well, and the incubation was continued for 3 h. The absorbance of the samples was measured at 440 nm with a reference wavelenght of 650 nm, against the controls (the same cells but not treated with gentamicin) using a microplate reader UVM 340 (Biogenet, Poland). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. This kit utilizes a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to produce 50% dismutation of superoxide radical. SOD activity was expressed in U/mg protein.

Catalase (CAT) assay

Catalase (CAT) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. This kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. One unit of CAT was defined as the amount of enzyme that causes the formation of 1.0 nM of formaldehyde per minute at 25°C. CAT activity was expressed in nM/min/mg protein.

Glutathione peroxidase (GPx) assay

Glutathione peroxidase (GPx) activity was measured using an assay kit (Cayman, MI, USA) according to the manufacturer's instruction. The measurement of GPx activity is based on the principle of a coupled reaction with glutathione reductase (GR). The oxidized glutathione (GSSG) formed after reduction of hydroperoxide by GPx is recycled to its reduced state by GR in the presence of NADPH. The oxidation of NADPH is accompanied by a decrease in absorbance at 340 nm. One unit of GPx was defined as the amount of enzyme that catalyzes the oxidation of 1 nM of NADPH per minute at 25°C. GPx activity was expressed in nM/min/mg protein.

Statistical analysis

In all experiments, the mean values of at least three separate experiments (n = 3) performed in triplicate ± standard error of the mean (SEM) were calculated. The results were analyzed statistically using GraphPad Prism 6.01 software. A value of $p < 0.05$ (*) or $p < 0.005$ (**), obtained with a Student's *t*-test by comparing the data with those for control (cells without gentamicin), was considered statistically significant.

RESULTS AND DISCUSSION

Although aminoglycosides induce ototoxicity and nephrotoxicity, they are still important antibiotics in current clinical practice and are widely used, especially in developing countries. Aminoglycosides exhibit broad-spectrum of antibacterial activity against enterococcal, mycobacterial, and especially multi-drug-resistant Gram-negative bacterial infections (2, 3). Additionally, aminoglycosides are commonly used in experimental ototoxicity models (28-30).

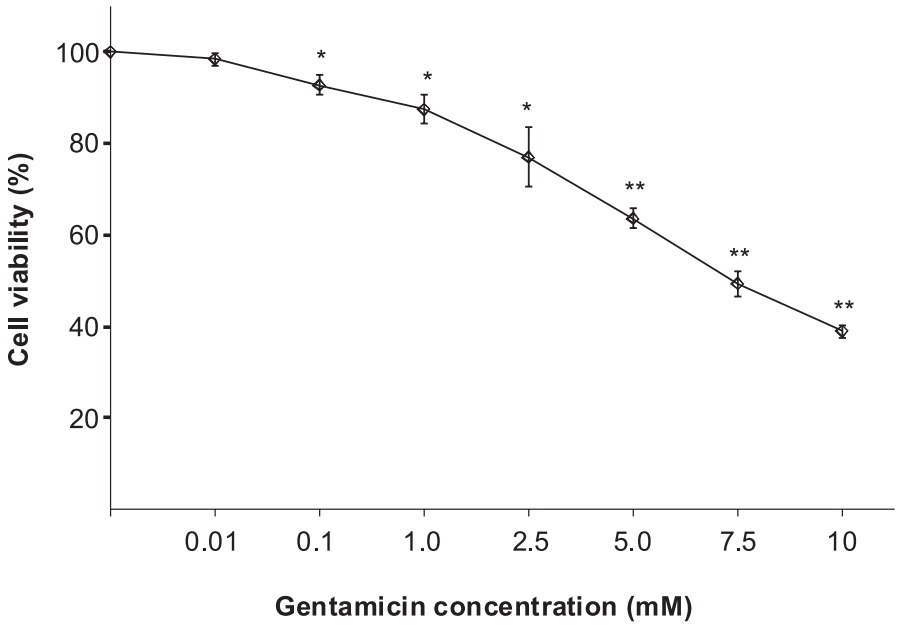

Figure 1. The effect of gentamicin on viability of melanocytes. Cells were treated with various concentrations of gentamicin (0.01-10 mM) and examined by WST-1 assay. Data are expressed as % of cell viability. The mean values ± SEM from three independent experiments (n = 3) performed in triplicate are presented. * $p < 0.05$ *vs.* the control samples; ** $p < 0.005$ *vs.* the control samples

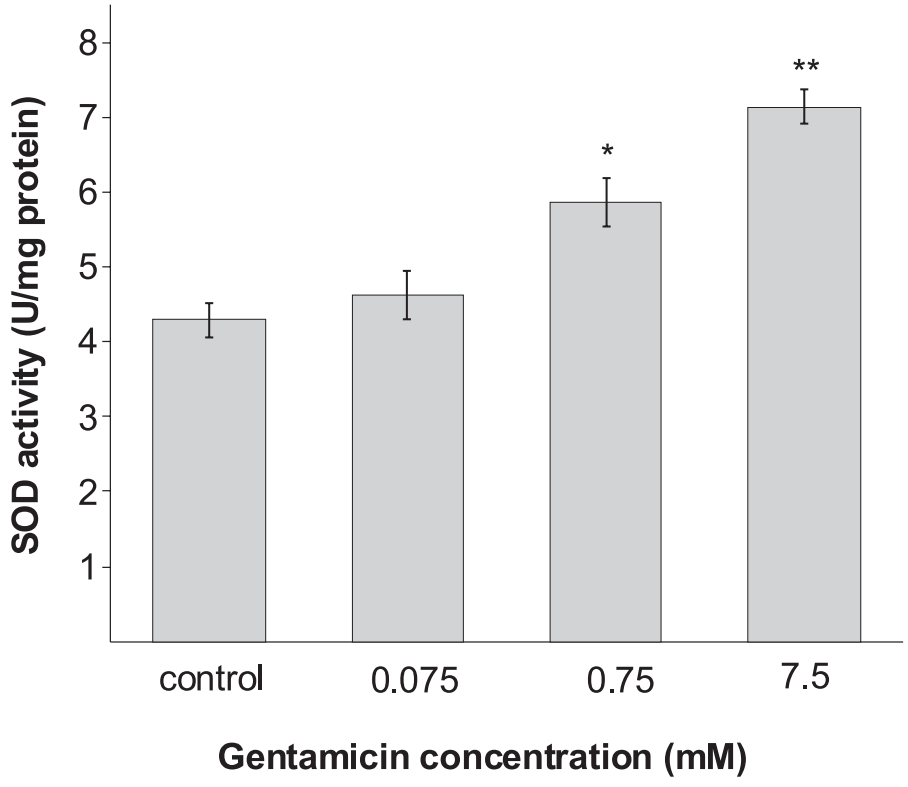

Figure 2. The superoxide dismutase (SOD) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean ± SEM of at least three independent experiments (n = 3) performed in triplicate. * $p < 0.05$ *vs.* the control samples; ** $p < 0.005$ *vs.* the control samples

Aminoglycoside-induced ototoxicity progresses from high to low frequencies with an increase in dose or duration of treatment (2). It has been suggested that the relationship between the aminoglycoside treatment and pigmented tissue damage is important to understand the ototoxic process. Many drugs are known to be markedly accumulated and retained for a considerable time by melanin-containing tissues and the retention of these compounds is proportional to degree of pigmentation. The ability of melanins to bind different drugs and transition metal ions is probably of the greatest biological importance (15).

The aim of the present study was to investigate the effect of gentamicin on antioxidant defense system in HEMn-DP melanocytes. Human melanocytes develop from the neural crest, later becoming distributed in the epidermis, hair bulbs of the skin, the uveal tract, the retinal pigment epithelium, the inner ear, and the leptomeninges, which are collectively regarded as pigmented organs. In this study, we have used the culture of normal human melanocytes as an *in vitro* experimental model system.

Melanocytes were treated with gentamicin in a range of concentrations from 0.01 mM to 10 mM for 24 h (Fig. 1). The cell viability was determined by the WST-1 test assay. At a relative low antibiotic concentration (0.01 mM) the loss in cell viability was not statistically significant. Cells treated with 0.1, 1.0, 2.5, 5.0, 7.5 and 10 mM of gentamicin for 24 h lost about 7.3, 12.5, 23.0, 36.3, 50.6 and 61.1% in viability, respectively. The value of EC_{50} (i.e., the amount of a drug that produces loss in cell viability by 50%) was 7.5 mM.

The ototoxic effects of aminoglycosides have been linked to oxidative stress. Aminoglycosides such as gentamicin can react with iron to generate ROS within the inner ear, with permanent damage to hair cells and neurons (2, 28, 31). Overproduction of ROS was suggested as an initial step in triggering apoptotic pathways, resulting in cell death due to aminoglycoside-induced ototoxicity. Methods of blocking ROS in the cochlea under *in vitro* or *in vivo* aminoglycoside exposure have been analyzed in many studies (31, 32). Although the ototoxicity caused by aminoglycosides is well documented, the molecular mechanism have not yet been precisely determined.

To explain the effect of the tested antibiotic on ROS metabolism, the activities of the antioxidative enzymes: SOD, CAT and GPx in melanocytes were estimated.

SOD converts superoxide radical anion ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2). In the presence of reduced transition metal ions (e.g., Fe^{2+} and Cu^{+}), hydrogen peroxide can be converted into highly reactive hydroxyl radical ($OH^{\bullet -}$). Alternatively, hydrogen peroxide may be converted into water by the enzymes catalase or glutathione peroxidase (33).

Human melanocytes HEMn-DP were exposed to gentamicin in concentrations of 0.075, 0.75 or 7.5 mM (EC_{50}) for 24 h. The first enzyme measured was the SOD, i.e., the enzyme which catalyzes the formation of hydrogen peroxide from superoxide anion. Gentamicin enhanced the SOD activity in a concentration-dependent manner (Fig. 2). Treatment of cells with 0.75 and 7.5 mM of gentamicin, increased the SOD activity by 37 and 66%, respectively, as compared with the controls. CAT and GPx work together to catalyze the breakdown of hydrogen peroxide, produced by SOD, to water. The intracellular CAT activity was significantly decreased by 24% for cells treated with gentamicin in EC_{50} concentration (7.5 mM). (Fig. 3). After gentamicin treatment in concentrations of 0.075 and 0.75 mM, no significant changes in cellular CAT activity were determined in comparison with the control cells (Fig. 3). The activity of GPx increased by 26% for melanocytes treated with gentamicin in concentration of 0.75 mM and decreased by 24% for cells treated with the antibiotic in concentration of 7.5 mM (EC_{50}), in comparison to the control cells (Fig. 4).

ROS are products of normal cells metabolism or xenobiotic exposure, and on concentrations, ROS can be beneficial or harmful to cells and tissues. At physiological low concentrations, ROS function as redox messengers in intracellular signaling and regulation while their excess induce oxidative modification of cellular macromolecules, inhibit protein function and promote cell death (33). A protective effect against drug-induced toxicity was provided by broad spectrum ROS scavengers, such as low molecular weight thiol compounds, vitamin E and salicylate (34). Melanin is known to be a scavenger of free radicals and it has been suggested that it possesses superoxide dismutase activity (35). Moreover, this biopolymer acts as a biochemical dustbin, mopping up potentially toxic agents (16). Such properties may be important for protecting the pigment cells as well as surrounding tissues from the natural toxins, xenobiotics, oxygen and ROS (including free radicals) (36). The ability of melanin to bind gentamicin used in low concentrations may prevent the antibiotic-induced toxic effects. The observed changes in antioxidant enzymes activity in cells exposed to the lowest gentamicin concentration (0.075 mM) confirmed that the antioxidative response is sufficient to compensate the increase in ROS formation. Under

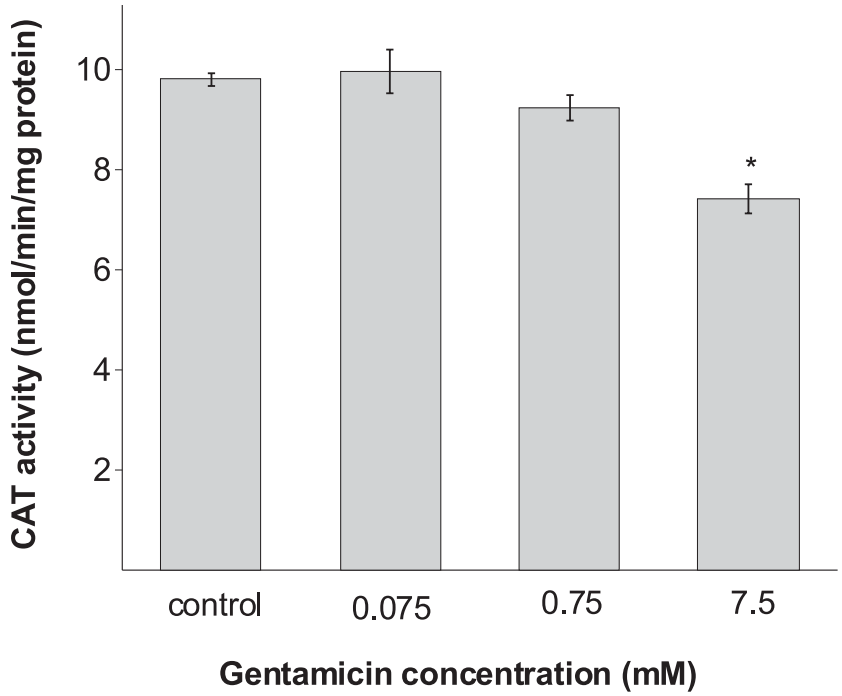

Figure 3. The catalase (CAT) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean ± SEM of at least three independent experiments (n = 3) performed in triplicate. * $p < 0.05$ *vs*. the control samples

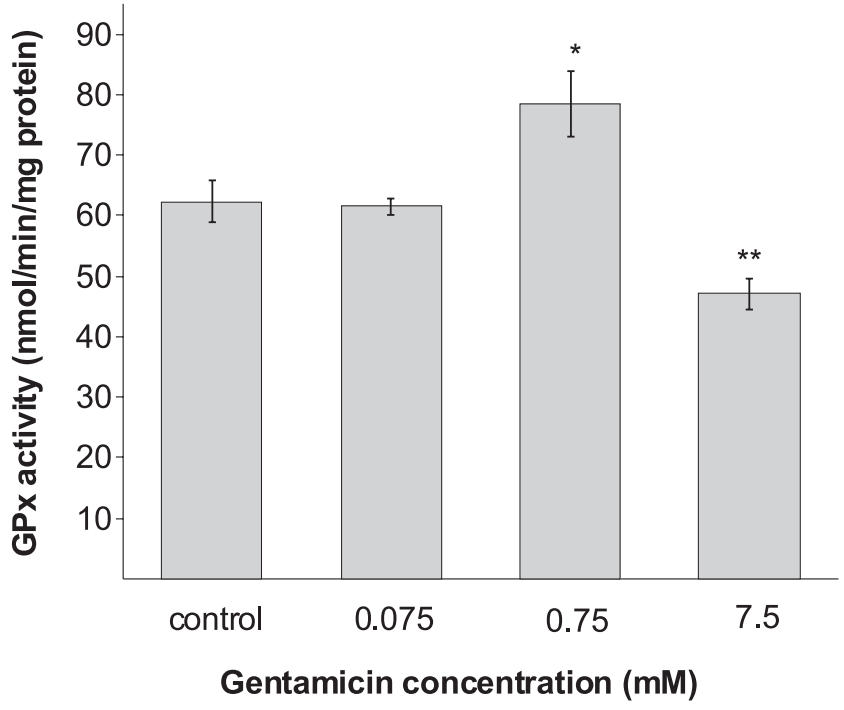

Figure 4. The glutathione peroxidase (GPx) activity in HEMn-DP cell after 24 h incubation with 0.075, 0.75 or 7.5 mM of gentamicin. Data are the mean ± SEM of at least three independent experiments (n = 3) performed in triplicate. * $p < 0.05$ *vs*. the control samples; ** $p < 0.005$ *vs*. the control samples

certain conditions, when ROS production is increased more strongly and persistently, the antioxidative response may not be sufficient to provide the cellular redox homeostasis (33). The use of gentamicin in higher concentrations (0.75 and 7.5 mM) induced depletion of antioxidant defense system and therefore it could be further concluded that the prolongation of treatment with the high concentration of gentamicin may augment the toxic effect of aminoglycosides on pigmented tissues of the inner ear.

Previously, we documented that other aminoglycoside antibiotics, namely kanamycin (25) and streptomycin (27) caused depletion of antioxidant status of light pigmented normal human melanocytes (HEMa-LP). The observed changes in antioxidant enzymes activity were much more higher than in HEMn-DP cells under gentamicin treatment. The results of our prior studies (25, 27) and those described in this work reveal that the large amount of melanin present in dark pigmented melanocytes may prevent cells against the ROS induced toxic effects. In addition, this phenomenon could be confirmed by the lower gentamicin cytotoxicity (EC_{50} = 7.5 mM) in regard to kanamycin (EC_{50} = 6.0 mM) and streptomycin (EC_{50} = 5.0 mM), what may explain a protective role of melanin in the mechanisms of aminoglycosides ototoxic effects.

CONCLUSION

In summary, the present work provides the first *in vitro* study of the mechanisms involved in gentamicin-induced toxic effects on pigmented tissues using HEMn-DP melanocytes. Based on the obtained results concerning the effect of gentamicin on antioxidant enzymes activity in melanocytes, the potential role of melanin and melanocytes in the mechanisms of aminoglycosides toxic effects directed to pigmented tissues was determined, especially during high-dose and/or long-term therapy.

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METHOTREXATE AND MYOTREXATE INDUCE APOPTOSIS IN HUMAN MYOMA FIBROBLASTS (T hES CELL LINE) *VIA* MITOCHONDRIAL PATHWAY

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Abstract: Uterine leiomyomas (fibroids) are the most common benign tumors in women of reproductive age. Although the local application of low doses of methotrexate (MTX) is used as an effective treatment of the myomas, myotrexate could be a promising new drug. This study investigated the cytotoxic and apoptotic effects of both MTX and myotrexate in human fibroblasts derived from the uterine fibroids (T hES cell line). The myotrexate adduct is an aqueous solution of MTX and L-arginine. Cells were treated with a graded concentrations of both MTX and myothrexate (0.1-16 μM) for 24 h. The cytotoxicity was assayed by MTT test, apoptosis was evaluated by Annexin V-FITC assay and their possible role in apoptosis was determined by immnuflourescence. Both MTX and myotrexate induced apoptosis in T hES cells in a dose dependent manner ($p < 0.001$). Myotrexate significantly increased the percentage of AnnexinV positive cells, BAX/Bcl-2 ratio and subsequent caspase-3 activation compared to the MTX treated cells ($p < 0.05$). Both MTX or myotrexate treatment showed a diffuse staining of cytochrome c indicating its release from mitochondria to the cytosol, suggesting that their mechanisms of action most likely involves the mitochondrial apoptotic pathway.

Keywords: MTX, myotrexate, apoptosis, Bcl-2 family, cytochrome c

Uterine leiomyomas (fibroids) are the most common benign tumors in women of reproductive age (1). Surgery, in the form of myomectomy or hysterectomy, is currently the primary treatment for uterine fibroids and so these tumors cause hysterectomy in the 77% of cases (2). The initiating factors that lead to the development of fibroids are not well understood.

However, some evidence supports that ovarian steroids, such as estrogen and progesterone, are the important factors for leiomyoma growth (3). Over the past decade, analogs of GnRH (Gonadotropin Releasing Hormone) have been commonly used in the conservative treatment of fibroids. Continuous application of GnRH analogs reliably and reversibly suppress the gonadal function causing the condition similar to hypogonadotropic hypogonadism and affect the reduction of the myoma size. Some literature data indicate that their effect is achieved by causing apoptosis in myomas (3), but the role of apoptosis in uterine myomas is still unknown. Our preliminary clinical study showed that local application of low dose of methotrexate (MTX) on the eighth day of menstrual cycle, during three consecutive cycles, reduced the volume of uterine myomas (20-30%), decreased menstrual bleeding, improved hematological status of patients and most importantly reduced incidence of hysterectomy and other invasive methods. This approach did not cause menopause and its side effects and was able to diminish the use of hormonal substitution therapy (4). MTX is used in the treatment of numerous lymphomas, osteosarcomas, breast, ovarian, lung and urinary bladder cancers (5-10). Furthermore, it serves as an immunosuppressive agent in the treatment of rheumatoid arthritis (11) and for hiperproliferative epidermal cell disorders (psoriasis) (12) that are often associated with the increased angiogenesis (13). MTX competitively inhibits dihydrofolate reductase (DHFR)

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and it prevents folic acid's participation in the *de novo* synthesis of nucleic acids and proteins (14), especially during the S-phase of the cell cycle. Thus, it achieves the greatest toxic effect on rapidly dividing cells. The exact mechanism of action of MTX is not fully understood. Recently, Spurlock et al. have shown that MTX did not induce apoptosis directly, but through indirect cells stimulation that led to the cells induced sensitivity to apoptosis, possibly through mitochondrial or death receptor pathway through Jun N-terminal kinase (JNK)-dependent mechanisms (15). Moreover, Savion et al. showed that MTX was directly involvolved with BAX regulation, a molecule of Bcl-2 family group (16), and key regulators of the apoptotic processes (17). One of MTX's shortcomings is the ability of tumor cells to continually develop resistance to it, possibly due to the amplification of the DHFR gene, or gene mutation that impairs MTX's binding. Using computer programs (Discovery Studio Visualizer and Hyperchem), we identified that by binding to DHFR, MTX is placed into a large hydrophobic niche (18, 19) where is predominantly surrounded by amino acid residues of L-arginine in a 1 : 5 ratio with which produces hydrogen and Wan der Waals links which are most responsible for secondary and tertiary structure and hence activity of the substrate.

Based on this observation, in our laboratory we synthesized the adduct myotrexate as an aqueous solution of MTX and L-arginine. Combining the results of NMR spectroscopy, quantum mechanical modelling and molecular dynamics simulations we got 3D look of solvating myotrexate. The final results show that the drug compounds in solution are unaltered on a molecular level, but held each other with strong inter- and intra-molecular hydrogen bonding. Hydrogen bonds are particularly pronounced between the deprotonated carboxyl groups of MTX and guanidine group the three of the five molecules of L-arginine.

Here, we investigated the effect of myotrexate on the basic cell component of myomas fibroblasts. Additionally, knowing that Bcl-2 group molecules play a role in the MTX apoptotic signalling pathway, we invstigated if that the same would apply to myotrexate.

Myotrexate has been classified as A 61 K 31/195 (WIPO Patent Application WO/2003/022260) by the International Patent Office.

The aim of this study was to investigate and compare both the cytotoxic and apoptotic effects of methotrexate and myotrexate and their potential role in apoptosis.

MATERIALS AND METHODS

Cell line

T hES cell line (T hES; ATCC®: CRL-4003tm) human fibroblasts derived from uterine fibroids, immortalized with human telomerase reversible transcriptase. Cells were grown in DMEM (Dulbecco's modified Eagle's medium, Sigma Aldrich, Germany) enriched with L-glutamine (2 mM/L, Invitrogen, USA), 1% nonessential amino acids (Sigma Aldrich, Germany), 1% ITS (insulin transferin supplement, BD Biosciences, USA), penicillin-streptomycin (1 mM/L, Sigma Aldrich, Germany) and 10% FBS (fetal bovine serum, Sigma Aldrich, Germany). Cells were incubated at 37°C, atmosphere of 5% CO_2.

Pharmaceutical composition (myotrexate) was prepared by dissolving 10 g of L- arginine in a 600 cm^3 of distilled water and the vessel was softly shaked until the L-arginine was completely dissolved. After that, 5 g of methotrexate was added and the vessel was softly shaked until all methotrexate was dissolved, and following that another 385 cm^3 of distilled water was added and the obtained solution was autoclaved. An aqueous solution of methotrexate and L-arginine prepared in this manner contains methotrexate and L-arginine in molar ratio of 1 : 5.

Cells were treated with pre-sterilized methotrexate (Sigma Aldrich, Germany) and/or myotrexate in concentrations of 16, 12, 8, 4, 2, 1, 0.1 and 0.01 μM and L-arginine (Sigma Aldrich, Germany) in concentrations 80, 60, 40, 20, 10, 5 and 0.5 μM.

Cytotoxic effect of these compounds was examined by MTT cytotoxicity assay.

Briefly, in a 96-well microtiter plate, cells were resuspended in a medium (1.8×10^4 cells/200 μL medium), treated with tested substances (MTX, myotrexate and L-arginine in appropriate concentrations for 24 h), and incubated with MTT solution (5 mg/mL MTT dissolved in PBS) for 4 h (37°C, 5% CO_2). After centrifugation (1000 rpm, 5 min) and removal of supernatant, cells were resuspended in 200 μL DMSO (Sigma Chemical, St. Louis, MO) per well and incubated for 30 min on a shaker, at room temperature, and away from light. The optical density (OD) was measured at wavelength 595 nm (multimode microplate detector, Zenuth 3100). The percentage of unviable cells was calculated by the formula: Cytotoxicity (%) = [1 - (experimental group (OD)) / (control group (OD)) × 100].

Investigation of apoptotic effect of these substances was performed by Annexin V-FITC assay.

Briefly, cells (5×10^5 cells/800 μL) were treated with tested substances (MTX, myotrexate and L-arginine in appropriate concentrations for 24 h), then washed two times with cold PBS, and resuspended in a binding buffer. Annexin V-FITC (5 μL) and propidium iodide (5 μL) was added in 100 μL of that solution. After 15 min, away from light, and at room temperature, 400 μL of binding buffer was added and cells were analyzed on FACS (Becton-Dickinson, FACS-Calibur, Montainview, CA, USA) within one hour.

The mechanism of apoptotic effect was determined by fluorescence microscopy.

Briefly, cells (2×10^4 cells/400 μL) were seeded on sterile cover slips and incubated for 24 h (37^oC, 5% CO_2) to achieve confluence of 80%. Cells were then treated with tested substances (MTX and myotrexate) for 24 h, washed in PBS, and fixed in 4% paraformaldehyde, 23 mM NaH_2PO_4 and 77 mM Na_2HPO_4 (pH 7.3). Cells were permeabilized for 10 min with 0.2% Triton-X/PBS, incubated for 30 min in a blocking buffer (10% FCS, 0.1% Triton X-100/PBS), washed and then incubated for one hour with different anti-rabbit primary antibodies: Bax (N20, sc-493, Santa Cruz Biotech. Inc.), Bcl-2 (DC21, sc-783, Santa Cruz Biotech. Inc.), caspase-3 (9661, Cell Signalling Technology, USA) and anti-mouse antibodies: cytochrome-c (G7421, Promega, USA), β-actin (A5316, Sigma Aldrich, Germany). After the incubation, cells were washed three times in PBS, incubated and stained with fluorescent secondary antibodies Alexa 488 (11001, Invitrogen, USA) and Cy3 (C7604, Sigma Aldrich, Germany) for one hour away from light at room temperature. Fluorescence of the cells was observed at 100× and 400× magnifications on Olympus (model BX51). ImageJ was used for image analysis.

Student's *t*-test or nonparametric Mann-Whitney's sum rank tests were used for statistical analysis. Results were analyzed by commercial software package SPSS (version 13) and are presented as the mean ± SD (standard deviation) and statistical significance was determined by the level of $p < 0.05$.

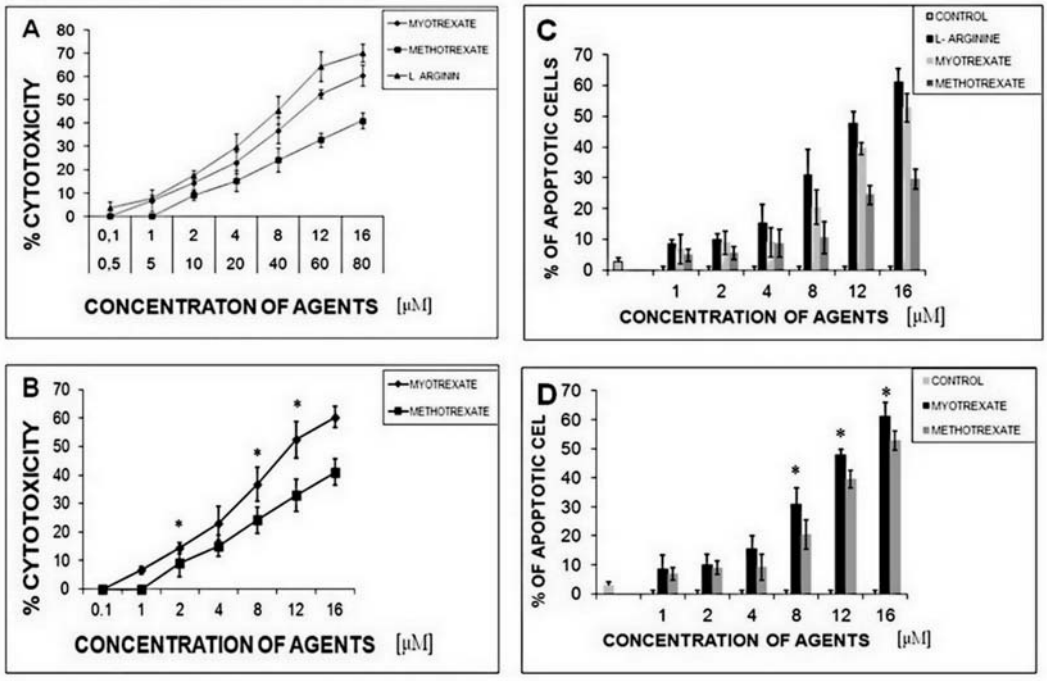

Figure 1. Cytotoxic and apoptotic effects of MTX, myotrexate and L-arginine on fibroblasts. Cells were treated with various concentrations of MTX, myotrexate and L-arginine for 24 h at 37°C. A) represent cytotoxic effect of those agents and B) comparison of cytotoxic effect of MTX and myotrexate. Cell viability was determined by MTT assay. Results are presented as the mean values of five independent experiments for each concentration ± standard deviation (SD). Statistical analysis of the presented relationships was performed with Student's *t* test for independent samples and statistical significance was determined by the level of $p < 0.05$. C) represent apoptotic effect of those agents and D) comparison of apoptotic effect of MTX and myotrexate. FACS analysis was performed with Annexin V-FITC and propidium-iodide. Results are presented as the mean values of three independent experiments for each concentration ± SD. Statistical analysis of the presented relationships was performed with Student's *t* test, the Mann-Whitney's test for independent samples, and statistical significance was determined by the level of $p < 0.05$

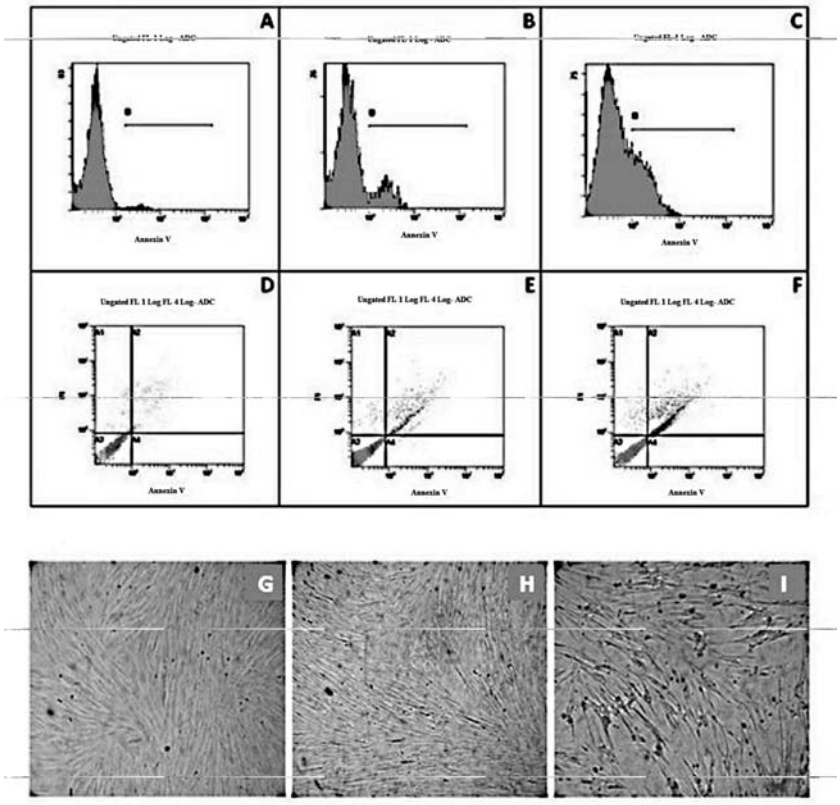

Figure 2. Phenotypic changes associated with fibroblasts following the treatment. Phenotypic changes associated with fibroblasts following 16 μM MTX and 16 μM myothrexate treatment are shown. Top panels depict FACS analysis of apoptosis of fibroblasts. A) and D) show apoptosis of untreated cells (spontaneous apoptosis); B) and E) show apoptosis of cells treated with MTX, C) and F) show apoptosis of cells treated with myotrexate. Bottom panel depicts morphological changes following those treatments. G) shows monolayer of untreated cells, H) MTX-treated, and I) myotrexate-treated. Morphological changes observed are associated with apoptosis. Cells were observed under the magnification of 40×

RESULTS

The cytotoxic effects of methotrexate (MTX), myotrexate and L-arginine on myoma fibroblasts

To investigate the cytotoxic activities of the MTX, myotrexate and L-arginine on cultured myoma fibroblasts, T hES cells were treated for 24 h with graded concentrations of those drugs (concentration range from 0.01 μM to 16 μM *vs.* from 0.5 μM to 80 μM) and the number of viable myoma cells was determined by MTT assay. Differences in the number of viable cells between the treated groups and the cells grown in complete medium were statistically significant ($p < 0.05$). MTX, myotrexate and L-arginine significantly decreased the cell numbers compared to control group (untreated cells) in dose dependent manner ($p < 0.001$). It was noted that L-arginine exerted the greatest cytotoxic effect on those cells. Cells treated with myotrexate at concentrations of 2, 8, 12, and 16 μM showed statistically significant decrease in the number of viable myoma cells compared to the cells treated with the same concentrations of MTX ($p < 0.05$); hence, myotrexate exerted the greater cytotoxic effect on T hES cells compared to methotrexate (Fig. 1).

MTX, myotrexate and L- arginine induce apoptosis in myomas fibroblasts

Since MTX, myotrexate and L-arginine demonstrated the cytotoxic effects on myoma fibroblasts, next we investigated the molecular type of cell death induced in these cells by those agents. Thus, T hES cells were treated with graded concentrations of MTX and myotrexate (from 1 to 16 μM) and L-arginine (from 5 to 80 μM) for 24 h and apoptosis was assessed with Annexin V/propidium iodide assay by flow cytometry. The apoptotic cells were defined as any cells colocalized with Annexin V in T hES cells. Induction of apoptosis by MTX,

myotrexate and L-arginine in T hES cells was statistically higher than in the control cells in a dose dependent manner ($p < 0.001$). L-arginine had the greatest effect of apoptosis on this cell line. Furthermore, myotrexate treatment of T hES cells, particularly at the concentrations of 8, 12 and 16 μM, significantly increased the percentage of AnnexinV positive cells compared to the MTX treated cells (Fig. 1).

Moreover, by examining the morphology of T hES cells treated with both agents at the concentration of 16 μM, we observed typical apoptotic morphological changes, such as cell rounding, condensation and detachment of cells compared to control. Here, myotrexate displayed stronger apoptotic morphological changes in the cells compared to MTX treated cells (Fig. 2). These results suggest myotrexate to be more efficient apoptotic agent on the cultured myoma fibroblasts than MTX.

MTX and myotrexate induce the decrease of Bcl-2 expression

Cell survival is enabled by anti-apoptotic protein Bcl-2 through the inhibition of apoptosis. The effects of different cytotoxic stimuli that induce apoptosis in the cells also lead to the reduction of the expression level of Bcl-2. Thus, we examined the effects of MTX and myotrexate stimulation of T hES cells on the total expression levels of endogenous Bcl-2 by immunoflourescence. T hES cells treated either with 8 μM concentration of MTX or myotrexate demonstrated significantly reduced expression of Bcl-2 compared to untreated cells by 19.54 ± 11.57% in MTX and by 71.35 ± 8.7% in myotrexate ($p < 0.05$; Fig. 3).

MTX and myotrexate treatment of myoma fibroblasts induces the activation of pro-apoptotic protein BAX

Apoptosis is regulated by Bcl-2 family members such as proapoptotic BAX that upon induction of apoptosis is activated and recruited into the outer bilayer of mitochondria causing its permeabilization. To investigate the possible role of BAX in our study, we tested the effect of MTX and myotrexate on BAX activation and its recruitment into the mitochondrial outer membrane by immunoflourescence. Our results demonstrated that both MTX and myotrexate at 8 μM

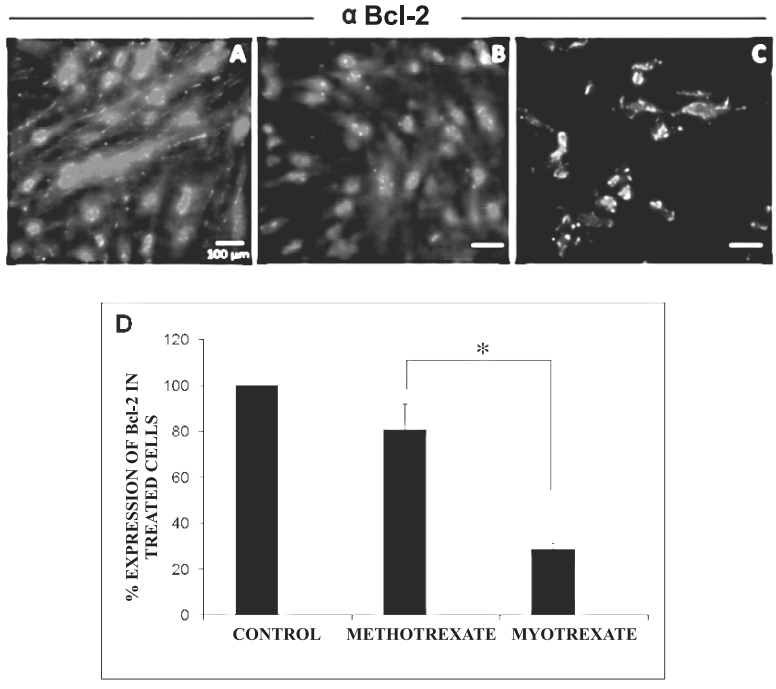

Figure 3. Total cell expression of anti-apoptotic protein Bcl-2 in the fibroblasts following MTX and myotrexate treatment. Cells were treated with 8 μM MTX and 8 μM myotrexate for 24 h and assayed for Bcl-2 expression. Top panel depicts immunofluorescent staining in A) untreated cells, B) cells treated with MTX, and C) cells treated with myotrexate. Cells were observed under 100× magnification. Bottom panel (D) represents quantified immunostaining results on the same number of cells in three different visual fields with ImageJ software. Cells treated with myotrexate demonstrated significantly reduced expression of Bcl-2 compared to MTX treated cells. Statistical analysis was performed with Student's *t* test for independent samples and statistical significance was determined by the level of $p < 0.05$

concentration induced the activation and incorporation of pro-apoptotic BAX into mitochondria of T hES cells compared to the control cells. Whereas the percent of cells expressing active BAX in cells treated with either MTX or myotrexate was 19.5 ± 3.4% and 32.42 ± 4.45%, respectively, the percent in untreated cells was only 3.19 ± 1.1% (Fig. 4). Therefore, both agents induced the activation of BAX; however, this effect was significantly greater in myotrexate treated cells compared to MTX treated cells.

MTX and myotrexate induce the release of cytochrome c from mitochondria

Following the stimulation of T hES cells with either MTX or myotrexate, activation and recruitment of BAX occurred into the mitochondrial outer membrane. Therefore, we further examined the effect of these drugs on the cytochrome c release from mitochondria. This was examined by immunoflourescence in both untreated cells and cells treated with MTX and myotrexate at the concentration of 8 μM (Fig. 5). T hES cells stimulated with either MTX or myotrexate showed diffuse staining of cytochrome c indicative of its release from mitochondria to the cytosol. However, the untreated T hES cells demonstrated a punctuate staining typical for the the cytochrome c localized in the intermembranous space of mitochondria.

MTX and myotrexate induce caspase-3 dependent apoptosis

To investigate the apoptotic mechanisms in the cells treated with MTX and myothrexate, next we analyzed the activation of caspase-3 by immunoflourescence. Our results demonstrated that both MTX and myothrexate treatment of the cells caused morphological changes in the cytoskeleton of fibroblasts as well as the activation of caspase-3, whereas the same changes were not detected in the untreated cells (Fig. 6). Percentage of cells expressing the active caspase-3 when stimulated with either MTX or myotrexate was 5.72 ± 2.05%, and 37.36 ± 5.3%, respectively ($p = 0.008$). The cell activation of caspase-3 in untreated samples was 3.41 ± 1.13%, a

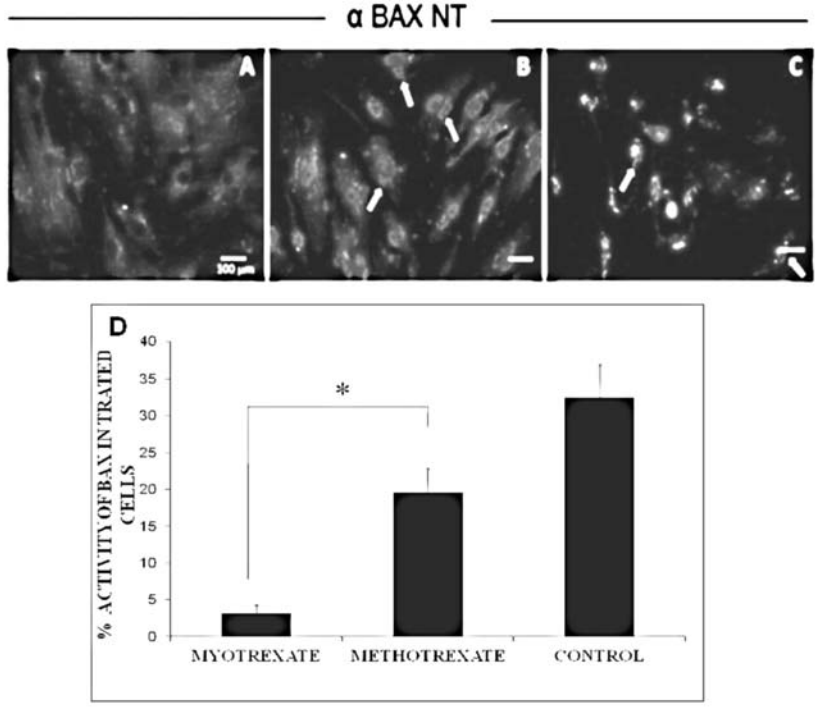

Figure 4. Activation of pro-apoptotic protein BAX following MTX and myotrexate treatment. ThES cells were treated with 8 μM methotrexate and 8 μM myotrexate for 24 h. Cells were then immunostained and signal quantified. Top panel represents immunostaining of active form of pro-apoptotic BAX so that in A) are untreated cells, B) cells treated with MTX, and in C) cells treated with myotrexate. Cells were observed under magnification 100×. Bottom panel (D) represents quantified immunostaining results on the same number of cells in three different visual fields with ImageJ software. The activation of BAX was significantly greater in myotrexate-treated cells compared to MTX-treated cells. Statistical analysis was performed with Student's *t* test for independent samples and statistical significance was determined by the level of $p < 0.05$

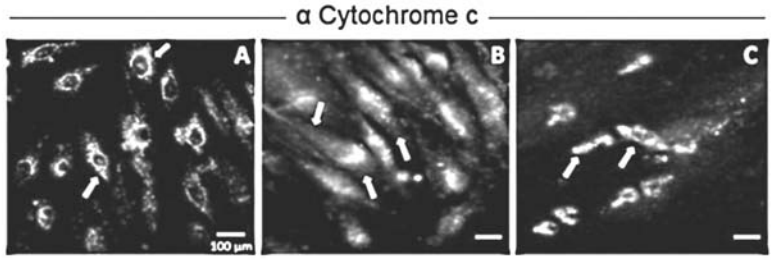

Figure 5. Release of cytochrome c from intramembranous space of mitochondria into the cytosol of fibroblasts. Cells treated with 8 µM MTX and 8 µM myotrexate for 24 h were analyzed for cytochrome c release by immunofluorescence. A) shows untreated cells, B) cells treated with MTX, and C) cells treated with myotrexate. Cells were observed under the 100× magnification

consequence of spontaneous apoptosis. Caspase-3 activation in cells treated with myotrexate was statistically significantly higher ($p < 0.05$) when compared to the MTX treated cells.

DISCUSSION

Although methotrexate (MTX) is commonly used in the treatment of numerous neoplastic and hiperproliferative disorders, our preliminary clinical study showed that local application of low dose of this antimetabolite agent could be used for myomas treatment as well. However, growing resistance to MTX has been reported for various cancers (20, 21). To overcome the resistance we sought to investigate the possibility of improving MTX's effects. Using computer programs, we identified that MTX binds L-arginine through hydrogen and Wan der Waals forces in a 1 : 5 ratio inside a hydrophobic pocket of the DHFR enzyme. Thus, we synthesized myotrexate, an aqueous solution of MTX and L-arginine and tested its efficacy on T hES cell line.

Analyzing the percentage of apoptotic cells, it was observed that the adduct myotrexate exhibited greater apoptotic effect compared to MTX and the statistical significance of this difference rises with increasing concentrations of these substances. Spectrophotometric analysis of myotrexate showed that MTX created strong hydrogen bonding with three molecules of L-arginine leading to its conformational change. As the concentration increases, differences in their apoptotic effect also increase, so might be assumed that two molecules of L-arginine, which are free and the number of which increases with rising concentration, contribute to the better apoptotic effect of myotrexate. MTX, which is in clinical use, is actually the sodium salt of MTX. By replacing NaOH with L-arginine to make it soluble (because MTX is insoluble in water) we have contributed to the improvement of its efficiency.

L-arginine is a basic semi-essential amino acid serving as a precursor for many important molecules for cellular physiology (proline, glutamate, creatine, nitric oxide (NO) and polyamines), making L-arginine one of the most versatile amino acids (22). The plasma concentration of L-arginine is about 200 µmol/L in humans (23). In some cell types, L-arginine, NO, and polyamines stimulate cell proliferation and reduce apoptosis (24) and inhibit cell proliferation and promote apoptosis in others (25, 26). Chen et al. have shown that supraphysiological concentration of L-arginine led to apoptosis of human choriocarcinoma cells (cell line JAR) (27) but this study have shown that L-arginine added to the culture media at subphysiological concentrations induced apoptosis in T hES cells as well.

Methotrexate's immunosuppressive, anti-inflammative, anti-proliferative and cytotoxic effects are consequences of apoptosis (28, 29).

Some studies have shown that MTX could induce apoptosis through Fas / Fas- ligand system (30) and the others demonstrated that it activated apoptosis *via* the mitochondrial pathway (27). Morphological changes, including a notable decrease in cell size, increase in granularity - phenomena that may implicate apoptotic death, cell shrinkage and cytoplasmic condensation as well as Annexin V test showed that apoptotic process was notable in T hES cells after treatment by MTX and myotrexate. In cells treated with tested substances, the release of cytochrome c from intermembranous space of mitochondria into the cytosol was observed, suggesting that apoptosis in these cells was realized through, so called, internal, mitochondrial pathway. After release into the cytoplasm, cytochrome c forms a complex with an enzyme called apoptosis activating factor-1 (Apaf-1) with consumption of energy (ATP). The complex binds to caspase-9, forming the apoptosome - oligomeric complex of cytochrome c/Apaf-1/cas-

pase-9. This complex primarily activates caspase-9, then binds and activates the effector caspase-3 and -7 (31). Activation of effector caspase-3 further causes the splits of actin fibers, subsequently leading to the formation of apoptotic bodies confirming that cells are succumbed to irreversible apoptotic process. The statatistically significant increase in Annexin V positive cells, levels of activated caspase-3 and pronounced morphological changes in myotrexate treated cells indicated a stronger apoptotic effect of this agent compared to MTX. One of the key regulators of the apoptotic process is the Bcl-2 family proteins consisting of two opposing groups: death antagonists (Bcl-2, Bcl-XL, Mcl-1) and death agonists (Bax, Bak, Bcl-XS) (32) so, cell apoptosis depends on the

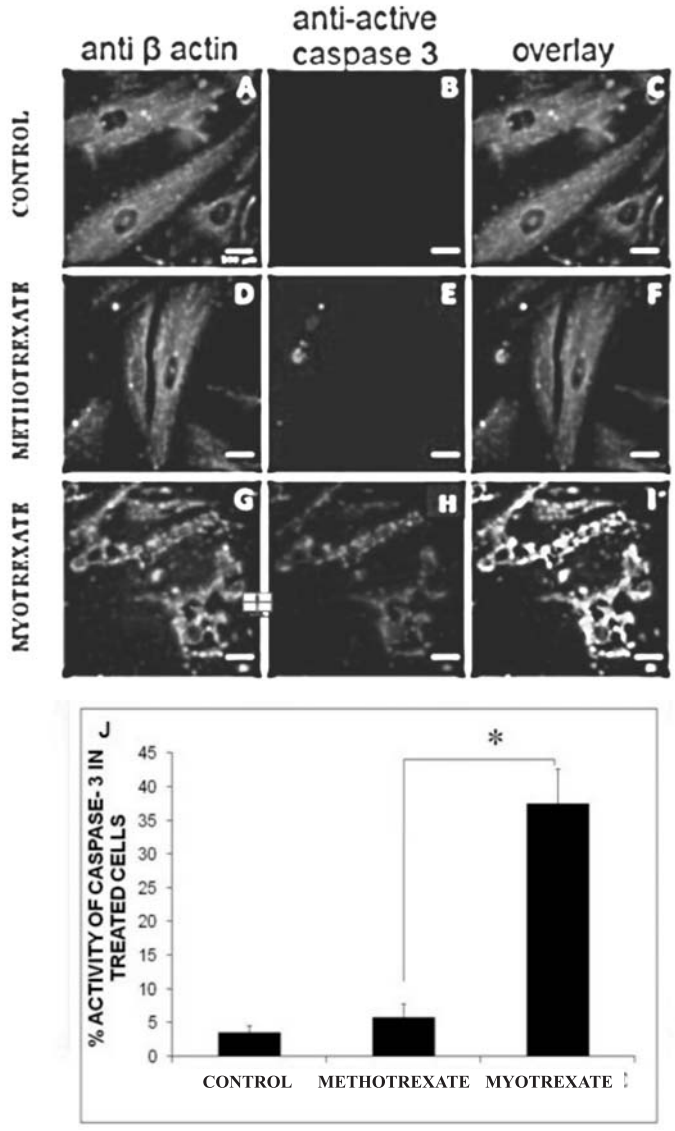

Figure 6. Activation of effector caspase-3 apoptotic protein following MTX and myotrexate treatment. Cells were treated with 8 μM MTX and 8 μM myotrexate for 24 h and assayed for caspase-3 activation and their morphological changes by immunofluorescence. On top figure, panels (A-C) depict unterated cells, (D-F) cells treated with MTX, and (G-I) cells treated with myotrexate. Left column (A, D, G) was stained with β-actin, middle column (B, E, H) was stained for anti-active caspase-3, and left column (C, F, I) present an overlay of those two. Cells were observed under 400× magnification. Bottom figure (J) represents quantified immunostaining results on the same number of cells in three different visual fields with ImageJ software. Caspase-3 activation in cells treated with myotrexate was statistically significantly higher compared to the MTX treated cells. Statistical analysis was performed using the Mann-Whitney's test for independent samples and statistical significance was determined by the level of $p < 0.05$

ratio of these protein groups (33). However, the exact mechanism of apoptosis regulation by Bcl-2 family is still unknown and being intensively investigated (34, 35). The fibroid cells have demonstrated increased Bcl-2 protein expression compared to the normal myometrial cells of the same uterus and the enlarged expression of Bcl-2 protein in leiomyoma cells is the molecular base for the increased proliferation of myocytes similar to the smooth-muscle cells of myometrium (36).

The influence of sex hormones (estrogen and progesterone) on the expression of pro- and anti-apoptotic proteins is well known (37-39). The treatment of myomas with GnRH was based on the indirect influence on these proteins and predominantly by reducing the level of sex hormones (40). However, in this *in vitro* investigation we presented a completely different approach to the treatment of fibroids by direct action on Bcl-2 protein family. Our results showed that MTX and myotrexate reduced the level of anti-apoptotic (Bcl-2), increased the level of pro-apoptotic proteins and the increase in BAX/Bcl-2 ratio led cells to apoptosis. Myotrexate was more efficient with the increase of this ratio; therefore, it could be a better potential apoptotic agent. So far, there are no known literature data on the effects of MTX and myotrexate on T hES cell line.

CONCLUSION

The findings presented in this study indicated that MTX and myotrexate exhibited cytotoxic effect on myoma fibroblasts (T hES cell line) and this effect was achieved by the induction of apoptosis *via* caspase dependent mitochondrial pathway and due to an imbalance in the expression of pro-apoptotic and anti-apoptotic proteins.

Myotrexate exhibited greater apoptotic effect compared to MTX partially due to conformational changes and partially due to the effect of free molecules of L-arginine.

This study opens up the possibility for further *in vivo* investigations that could indicate that myotrexate might be a potential new drug not only for the conservative treatment of myomas, but also as a possibility of applying this drug in the treatment of the other disorders where MTX is commonly used.

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DRUG SYNTHESIS

SYNTHESIS AND *IN VITRO* ANTIMICROBIAL ACTIVITY OF NOVEL SERIES OF 3,5-DIACETYLPYRIDINE COMPOUNDS

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Abstract: Bis diacetylpyridine derivative (**1**) was prepared and reacted with different halo-compounds, namely: epichlorohydrine and dichloroethyl ethyl ether to give **2a**,**b**, respectively, and reacted with morpholine and piperidine to afford Mannich products **3a**,**b**, successively. Compound **4** was synthesized by reaction of **1** with potassium thiocyanate. Reaction of **4** with 4-chlorobenzaldehyde, glucose and phthalic or maleic anhydrides produced **5**, **6** and **7a**,**b**. Compound **1** reacted with 4-chlorobenzaldehyde to give bisanylmethylene derivative **8**. Also some new compounds **9**-**11** were prepared from the reaction of compound **8** with nucleophiles, namely: hydrazine hydrate, thiosemicarbazide and hydroxylamine *via* Michael condensation reaction. On the other hand, compound **8** was reacted with cyclohexanone and cyclopentanone to give **12a**,**b**. The structures of newly synthesized products have been deduced on the basis of elemental analysis and spectral data. Some synthesized compounds were screened for their antimicrobial evaluation. Among the assayed compounds, derivatives **3b** and **12a** showed the highest antimicrobial activities.

Keywords: bis diacetylpyridines, aminothiazoles, pyrazoles, oxazoles, antimicrobial evaluation

1,4-Dihydropyridine is a six membered aromatic ring containing N atom at the 1st position and is saturated at the 1st and 4th positions. Literature survey exhibits that the pyridine derivatives possess wide spectrum of biological activities such as the calcium channel antagonistic effect (1), antianginal (2-4), antitumor (5), anti-inflammatory (6, 7), antitubercular (8), analgesic activity (9), antithrombotic (10, 11), vasolidation (12), anticonvalsant (13) and stress protective (14). Also, various pyridine derivatives have been synthesized as insecticides (15, 16), antifungal (17), antibacterial (18), herbicidal (19) and antimicrobial agents compared to oxytetracycline (20). Many studies have been devoted to the photochemistry and photooxidation of symmetrical dihydropyridine drugs such as lacidipine (21), nifedipine (22-26) and unsymmetrical dihydropyridine such as amlodipine (27), nisoldipine (28), nilvadipine (29) and nimodipine (30). As regards biological implications, thiosemicarbazide complexes have been intensively investigated for their antiviral, anticancer, antitumor, antimicrobial, antiamoebic and anti-inflammatory activities (31-41). This information encouraged us to synthesize new pyridine compounds to evaluate their antimicrobial activity against different strains of Gram positive, Gram negative bacteria and fungi.

EXPERIMENTAL

Chemistry

All melting points are uncorrected and were recorded in open glass capillary tubes using an Electrothermal IA 9100 digital melting point apparatus. Elemental microanalyses were carried out at Micro Analytical Unit, Central Service Lab (CSL), National Research Centre (NRS), using Vario Elementar apparatus and were found within ± 0.4% of the theoretical values. IR spectra were recorded on Jasco FT/IR, Fourier Transform, infrared spectrometer (Japan), while ^{1}H- and ^{13}C-NMR spectra were obtained using JEOL EX-270 and 500 using available solvent and TMS as internal standard. Mass spectra were recorded on Finnigan Mat SSQ-7000 mass spectrometer at CSL, NRS. TLC on silica gel-60, F254, aluminum sheets were also used.

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4-(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis-(1-oxidanylideneethyl)-1,4-dihydropyridine (1)

A mixture of 4-chlorobenzaldehyde (0.01 mol), acetylacetone (0.02 mol) and 1 g ammonium acetate in 30 mL H_2O was refluxed for 6 h. The solid formed was filtered off and crystallized from diethyl ether.

Yield: 60%; m.p. 180-182°C. IR (KBr, cm^{-1}): 3153 (NH), 1700, 1703 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 2.30 (s, 6H, $2CH_3$), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz, 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H), 9.71 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 8.70 ($2CH_3$), 23.10 ($2CH_3$), 26.40 (CH), 109.60 (2C=C), 128.30-135.80 (6 Ar-C), 140.40 (2C=C), 196.50 (2C=O). MS *m*/*z* (%): 303 (100), 192 (70). Analysis: calcd. for $C_{17}H_{18}ClNO_2$ (303.78): C, 67.21, H, 5.97, N, 4.61%; found: C, 67.00; H, 5.93, N, 4.65%.

General procedure for synthesis of compounds 2

A mixture of compound **1** (3.03 g, 0.01 mol) and sodium hydroxide (0.80 g, 0.02 mol) in ethanol (20 mL) was stirred at 60°C for 3 h. The reaction mixture was cooled and then epichlorohydrine or dichloroethyl ethyl ether (0.02 mol) was added. The reaction mixture was heated under reflux for 3 h, then evaporated. The residue was washed with H_2O, filtered off and recrystallized from ethanol.

4-(4-Chloranylphenyl)-2.6-dimethyl-3,5-bis(1-oxidanylideneethyl)-1-(oxiran-2-yl-methyl)-1,4-dihydropyridine (2a)

Yield: 52%; m.p. 162-164°C. IR (KBr, cm^{-1}): 1698, 1703 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$, acetyl), 2.30 (s, 6H, $2CH_3$, acetyl), 2.51 (m, 2H, CH_2, oxiranyl ring), 2.77 (m, 1H, CH-oxiranyl ring), 2.80 (m, 2H, CH_2), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz, 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d_6, δ, ppm): 16.50 ($2CH_3$), 23.00 ($2CH_3$), 26.70 (CH-pyroline), 44.61 (CH_2-oxiranyl ring), 50.30 (CH, oxiranyl ring), 52.11 (CH_2), 109.60 (2C=C), 128.80-135.80 (6 Ar-C), 140.40 (2C=C), 196.50 (2C=O). MS *m*/*z* (%): 359 (50), 303 (100). Analysis: calcd. for $C_{20}H_{22}ClNO_3$ (359.84): C, 66.75, H, 6.16, N, 3.89%; found: C, 66.80, H, 6.20, N, 4.10%.

1-(2-[(2-Chloranylethyl)oxidanyl]ethyl)-4-(4-chloranylphenyl)-2,6-dimethyl-3,5-bis(1-oxidanylideneethyl)-1,4-dihydropyridine (2b)

Yield: 51%; m.p. 126-128°C. IR (KBr, cm^{-1}): 1690, 1700 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$), 2.30 (s, 6H, $2CH_3$), 2.8 (t, 2H, CH_2N), 3.51 (t, 2H, CH_2O), 3.55 (t, 2H, CH_2Cl), 3.61 (t, 2H, CH_2O), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz, 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H), 9.71 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 410 (70), 303 (100). Analysis: calcd. for $C_{21}H_{25}Cl_2NO_3$ (410.33): C, 61.47, H, 6.14, N, 3.41%; found: C, 61.44, H, 6.14, N, 3.50%.

General procedure for synthesis of compounds 3

Formaldehyde (1 mL, 40%) was added to compound **1** (3.03 g, 0.01 mol) in dry ethanol (30 mL), and the reaction mixture was heated for 5 min, cooled, then secondary amine, morphine or piperidine (0.02 mol) was added and the reaction mixture was stirred overnight at room temp. The formed solid was filtered off, dried and recrystallized from methanol.

4-[(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(1-oxidanylideneethyl)pyridine-1-(4H)-yl)]-morpholine (3a)

Yield: 67%; m.p. 173-175°C. IR (KBr, cm^{-1}): 1698, 1703 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.66 (s, 6H, $2CH_3$), 2.30 (s, 6H, $2CH_3$), 2.37 (t, 4H, morpholine-H), 3.67 (t, 4H, morpholine-H), 3.95 (s, 2H, N-CH_2-N), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H). ^{13}C NMR (DMSO-d_6, δ, ppm): 16.20 ($2CH_3$), 23.00 ($2CH_3$), 26.70 (CH-pyridine), 54.70 (2C-morpholine), 69.70 (CH_2), 71.5 (2C-morpholine), 109.00 (2C=C), 128.80-135.60 (6 Ar-C), 140.40 (2C=C), 196.50 (2C=O). MS *m*/*z* (%): 402 (70), 303 (100). Analysis: calcd. for $C_{22}H_{27}ClN_2O_2$ (402.91): C, 65.58, H, 6.15, N, 6.92%; found: C, 65.66, H, 6.75, N, 7.00%.

4-[(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(1-oxidanylideneethyl)-1-(piperidin-1-yl-methyl)]-1,4-dihydropyridine (3b)

Yield: 65%; m.p. 121-123°C. IR (KBr, cm^{-1}): 1698, 1703 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.17-1.49 (m, 6H, piperidine-H), 1.70 (s, 6H, $2CH_3$), 2.30 (s, 6H, $2CH_3$), 2.20-2.45 (m, 4H, piperidine-H), 3.72 (s, 2H, N-CH_2-N), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz 2H, Ar-H), 7.15 (d, *J* = 9 Hz 2H, Ar-H). MS *m*/*z* (%): 400 (80), 303 (100). Analysis: calcd. for $C_{23}H_{29}ClN_2O_2$ (400.92): C, 68.90, H, 7.29, N, 6.99%; found: C, 68.00, H, 7.32, N, 6.98%.

3,5-Bis(2-amino-1,3-thiazol-5-yl)-4-(4-chloranylphenyl)-2,6-dimethyl-1,4-dihydropyridine (4)

A mixture of compound **1** (3.03 g, 0.01 mol) and potassium thiocyanate (1.94 g, 0.02 mol) was refluxed in glacial acetic acid containing 4 mL

bromine for 3 h. The reaction mixture was cooled and poured into ice water. The formed solid was filtered off, dried and crystallized from dioxane.

Yield: 60%; m.p. 202-204°C. IR (KBr, cm^{-1}): 3350 (NH_2), 3240 (NH). ^1H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$), 4.00 (s, 4H, $2NH_2$, D_2O exchangeable), 4.43 (s, 1H, pyridine-H), 7.00 (d, *J* = 9 Hz, 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H), 7.50 (s, 2H, thiazole-H), 9.8 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.80 ($2CH_3$), 43.40 (CH-pyridine), 107.80 (2C=C), 108.00 (2C=C), 128.80-130.60 (6Ar-C), 130.90 (2C=C), 139.00 (2CH), 172.00 (2C=N). MS *m*/*z* (%): 415 (85), 304 (100). Analysis: calcd. for $C_{19}H_{18}ClN_5S_2$ (415.96): C, 54.86, H, 4.36, N, 16.84%; found: C, 54.80, H, 4.40, N, 16.80%.

3,5-Bis(4-chloranylbenzylidene)amino-1,3-thiazole-5-yl)-4-(4-chloranylphenyl)-2,6-dimethyl-1,4-dihydropyridine (5)

A mixture of compound **4** (4.15 g, 0.01 mol) and 4-chlorobenzaldehyde (5.60 g, 0.02 mol) in acetic anhydride (30 mL) was refluxed for 11 h. The solution was cooled, poured into cold water and the precipitate formed was crystallized from glacial acetic acid.

Yield: 45%; m.p. 255-257°C. IR (KBr, cm^{-1}): 3230 (NH). ^1H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.43 (s, 1H, pyridine-H), 6.92-7.61 (m, 12H, Ar-H), 8.00 (s, 2H, thiazole-H), 8.10 (s, 2H, Schiff's base), 9.8 (s, 1H, NH, D_2O exchangeable). Analysis: calcd. for $C_{33}H_{24}Cl_3N_5S_2$ (660.64): C, 63.74, H, 4.25, N, 10.93%; found: C, 64.00, H, 4.50, N, 10.90%.

3,5-Bis[(2,3,4,5,6-pentahydroxyhexylidine) amino-1,3-thiazol-5-yl]-4-(4-chloranylphenyl)-2,6-dimethyl-1,4-dihydropyridine (6)

Compound **4** (4.15 g, 0.01 mol) and glucose (7.20 g, 0.2 mol) in ethanol (30 mL) containing 1 mL glacial acetic acid was heated with continuous stirring at 80°C for 6 h. The formed precipitate was filtered off, dried and recrystallized from ethanol.

Yield: 63%; m.p. 188-190°C. IR (KBr, cm^{-1}): 3451-3219 (br, OH and NH), 1590 (CH=N). ^1H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$), 3.32-3.92 (m, 12H glucose-H), 4.43 (s, 1H, pyridine-H), 4.19-5.00 (m, 10 H, OH, D_2O exchangeable), 6.94 (d, *J* = 9 Hz, 2H, Ar-H), 7.00 (d, *J* = 9 Hz, 2H, Ar-H), 7.40 (2H, CH=N), 8.00 (s, 2H, thiazole-H), 9.80 (1H, NH, D_2O exchangeable). Analysis: calcd. for $C_{31}H_{38}ClN_5O_{10}S_2$ (739.82): C, 53.39, H, 5.74; N, 9.73%; found: C, 53.52; H, 5.70, N, 9.70%.

General procedure for synthesis of compounds 7

To a solution of compound **4** (4.15 g, 0.01 mol) in acetic acid, phthalic anhydride or maleic anhydride (0.02 mol) was added. The mixture was refluxed for 8 h, then poured into ice water. The formed solid was filtered off, washed with water and recrystallized from dioxane.

4-(4-Chloranylphenyl)-3,5-bis[2-(2,7-dioxidanylidene-2,7-dihydroindolin-1-yl)-1,3-thiazol-5-yl]-2,6-dimethyl-1,4-dihydropyridine (7a)

Yield: 55%; m.p. > 300°C. IR (KBr, cm^{-1}): 3235 (NH), 1698 (2C=O), 1702 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.43 (s, 1H, pyridine-H), 7.10-8.20 (m, 12H, Ar-H + 2H, pyrazole-H), 9.82 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 676 (27). Analysis: calcd. for $C_{35}H_{22}ClN_5O_4S_2$ (676.17): C, 62.17, H, 3.28, N, 10.36%. found: C, 62.20, H, 3.30, N, 10.30%.

4-(4-Chloranylphenyl)-3,5-bis[2-(2,5-dioxidanylidene-2,5-dihydro-1H-pyrol-1-yl)-1,3-thiazol-5-yl]-2,6-dimethyl-1,4-dihydropyridine (7b)

Yield: 69%; m.p. 285-287°C. IR (KBr, cm^{-1}): 3230 (NH), 1701 (2C=O), 1705 (2C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.43 (s, 1H, pyridine-H), 6.12 (d, *J* =5.2 Hz, 2H, vinylic-H), 6.32 (d, *J* = 5.2 Hz, 2H, vinylic-H), 7.00 (d, *J* = 9 Hz 2H, Ar-H), 7.15 (d, *J* = 9 Hz, 2H, Ar-H), 7.50 (s, 2H, thiazole-H), 9.80 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 576 (60). Analysis: calcd. for $C_{27}H_{18}ClN_5O_4S_2$ (576.48): C, 56.30, H, 3.15, N, 12.20%; found: C, 56.33, H, 3.13, N, 12.25%.

4-[(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(4-chloroanylphenyl)-1-oxidanylideneprop-2-enyl]-1,4-dihydropyridine (8)

A mixture of compound **1** (3.03 g, 0.01 mol) and 4-chlorobenzaldehyde (2.24 g, 0.02 mol) in ethanol containing 1 g sodium hydroxide was refluxed for 3 h, then cooled and poured into water. The precipitate formed was filtered off and recrystallized from dioxane.

Yield: 50%; m.p. 162-164°C. IR (KBr, cm^{-1}): 3250 (NH), 1698 (C=O). ^1H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$), 4.45 (s, 1H, pyridine-H), 7.00-7.26 (m, 12H, Ar-H), 7.33 (d, *J* = 12.9 Hz, 2H, methylene), 7.96 (d, *J* = 12.9 Hz, 2H, methylene), 9.80 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 548 (85), 437 (100). Analysis: calcd. for $C_{31}H_{24}Cl_3NO_2$ (548.88): C, 67.83, H, 4.41, N, 2.55%; found: C, 67.87, H, 4.38, N, 2.60%.

4-(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(chloranylphenyl)-1H-pyrazol-3-yl)-1,4-dihydropyridine (9)

A mixture of compound **8** (5.48 g, 0.01 mol) and hydrazine hydrate (1 mL, 0.03 mol) was refluxed in absolute ethanol (30 mL) for 4 h. The precipitated solid was filtered off, and crystallized from methanol.

Yield: 45%; m.p. 204-206°C. IR (KBr, cm^{-1}): 3160, 3217, 3220 (3NH). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.42 (s, 1H, pyridine-H), 6.50 (s, 2H, pyrazole-H), 7.00-7.42 (m, 12H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable), 10.20 (s, 2H, NH-pyrazole, D_2O exchangeable). MS *m*/*z* (%): 572 (55), 461 (100). Analysis: calcd. for $C_{31}H_{24}Cl_3N_5$ (572.91): C, 64.99, H, 4.22, N, 12.22%; found: C, 65.20, H, 4.20, N, 22.40%.

3,5-Bis[1-(aminosulfanylidine)methyl-5-(4-chloranylphenyl)-1H-pyrazol-3-yl]-2,6-dimethyl-4-(4-chloranylphenyl)-1,4-dihydropyridine (10)

A mixture of compound **8** (5.48 g, 0.01 mol) and thiosemicarbazide (1.80 g, 0.02 mol) was refluxed in 30 mL ethanol containing sodium hydroxide (0.80 g, 0.02 mole) for 4 h. The reaction mixture was cooled, poured onto water and the formed solid was filtered off and recrystallized from dioxane.

Yield: 50%; m.p. 228-230°C. IR (KBr, cm^{-1}): 3150 (NH), 3360-3365 ($2NH_2$), 1228 (C=S). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 2.00 (s, 4H, $2NH_2$, D_2O exchangeable), 4.42 (s, 1H, pyridine-H), 6.50 (s, 2H, pyrazole-H), 7.00-7.62 (m, 12H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable). ^{13}C NMR (DMSO-d_6, δ, ppm): 18.80 ($2CH_3$), 43.40 (CH-pyridine), 104.00 (2CH, pyrazole), 107.80 (2C=C), 128.40-135.80 (18Ar-C), 130.70 (2C=C), 134.00 (2C=C), 150.00 (2C=C), 190 (2C=S). MS *m*/*z* (%): 691 (60), 580 (100). Analysis: calcd. for $C_{33}H_{26}Cl_3N_7S_2$ (691.09): C, 57.35, H, 3.79, N, 14.19%; found: C, 57.40, H, 3.80, N, 14.22%.

4-(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(4-chloranylphenylisoxazol-3-yl)-1,4-dihydropyridine (11)

A mixture of compound **8** (5.48 g, 0.01 mol) and hydroxyl amine hydrochloride (1.40 g, 0.02 mol) was refluxed in 30 mL pyridine for 3 h. The reaction mixture was cooled, poured into cold water and neutralized with dil. HCl. The formed solid was filtered off and recrystallized from acetic acid.

Yield: 45%; m.p. 150-152°C. IR (KBr, cm^{-1}): 3145 (NH). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.45 (s, 1H, pyridine-H), 6.55 (s, 2H, isoxazole-H), 7.00-7.45 (m, 12H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 574 (30), 463 (100). Analysis: calcd. for $C_{31}H_{22}Cl_3N_3O_2$ (574.88): C, 64.77, H, 3.86, N, 7.31%; found: C, 64.77, H, 3.90, N, 7.35%.

General procedure for synthesis of compounds 12

A mixture of compound **8** (5.48 g, 0.01 mol), cyclohexanone or cyclopentanone (0.04 mol) was stirred in 30 mL ethanol containing sodium hydroxide (0.06 mol) for 12 h at room temp. The mixture was extracted with ethyl acetate (20 mL) and dried over sodium sulfate anhydrous. After removing off the solvent *in vacuo*, the collected gummy product was precipitated in CCl_4/hexane (3 : 1) and crystallized from dioxane.

[4-(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(4-chloranylphenyl)-1-oxidanylidene-prop-2-enyl]-1,4-dihydropyridine]cyclohexanone (12a)

Yield: 45%; m.p. 107-109°C. IR (KBr, cm^{-1}): 1695, 1698 (2C=O), 1707-1710 (2C=O). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70 (s, 6H, $2CH_3$), 4.43 (s, 1H, pyridine-H), 1.80-2.59 (m, 18H, cyclohexanone), 3.17-3.20 (m, 2H, propyl-H), 3.25 (dd, *J* = 11.66, 2.60 Hz, 2H, propyl-H), 3.40 (dd, *J* = 12.90, 3.37 Hz, 2H, propyl-H,), 7.00-7.19 (m, 12H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 745 (70). Analysis: calcd. for $C_{43}H_{44}Cl_3NO_4$ (745.17): C, 69.31, H, 5.95, N, 1.88%; found: C, 69.40, H, 5.92, N, 2.00%.

[4-(4-Chloranylphenyl)-2,6-dimethyl-3,5-bis(4-chloranylphenyl)-1-oxidanylideneprop-2-enyl]-1,4-dihydropyridine]cyclopentanone (12b)

Yield: 61%; m.p. 110-112°C. IR (KBr, cm^{-1}): 1698, 1700 (2C=O), 1702, 1677 (2C=O). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71 (s, 6H, $2CH_3$), 2.06-2.43 (m, 14H, pentanone-H), 3.20-3.23 (m, 2H, propyl-H), 3.30 (dd, *J* = 11.88, 3.00 Hz, 2H, propyl-H), 3.45 (dd, *J* = 12.94, 3.25 Hz, 2H, propyl-H), 4.43 (s, 1H, pyridine-H), 7.00-7.19 (m, 12H, Ar-H), 9.80 (s, 1H, NH, D_2O exchangeable). MS *m*/*z* (%): 717 (90). Analysis: calcd. for $C_{41}H_{40}Cl_3NO_4$ (717.11): C, 68.67, H, 5.6,2 N, 1.95%; found: C, 68.70, H, 5.60, N, 1.90%.

Antimicrobial activity

The antibacterial activity of the synthesized compounds was tested against *Bacillus subtilis* NRRL 543 , *Staphylococcus aureus* NRRL B-313 (Gram-positive bacteria), *Escherichia coli* NRRL B-210, *Pseudomonas aeruginosa* NRRL B-23 (Gram-negative bacteria) using nutrient agar medium. The

antifungal activity of the compounds was tested against *Candida albicans* NRRL Y-477 *and Aspergillus niger* NRRL 599 using Sabouraud dextrose agar medium.

Agar diffusion medium

All compounds were screened *in vitro* for their antimicrobial activity by agar diffusion method (42). A suspension of the organisms were added to sterile nutrient agar media at 45°C and the mixture was transferred to sterile Petri dishes and allowed to solidify. Holes of 10 mm in diameter were made using a cork borer. An amount of 0.1 mL of the synthesized compounds was poured inside the holes. A hole filled with DMSO was also used as control. The plates were left for 1 h at room temperature as a period of pre-incubation diffusion to minimize the effects to variation in time between the applications of the different solutions. The plates were then incubated at 37°C for 24 h and observed for antibacterial activity. The diameters of zone of inhibition were measured and compared with that of the standard; the values were tabulated. Ciprofloxacin (50 µg/mL) and fluconazole (50 µg/mL) were used as standard for antibacterial and antifungal activity, respectively. The observed zones of inhibition are presented in Table 1.

Minimal inhibitory concentration

Minimal inhibitory concentration (MIC) of the test compounds were determined by agar streak dilution method. Stock solutions of the synthesized compounds (100 mg/mL) were made using DMSO as the solvent. From this stock solution, a range of concentrations from 5 to 0.05 mg/mL of the tested compounds solutions was mixed with the known quantities of molten sterile agar media aseptically. About 20 mL of nutrient agar medium for bacteria and Sabouraud dextrose agar medium for fungi containing the tested compound under study was dispensed into each sterile Petri dish. Then, the media were allowed to get solidified. Microorganisms were then streaked one by one on the agar plates aseptically. After streaking, all the plates were incubated at 30°C for 24 h/48 h for bacteria and fungi, respectively. Then, the plates were observed for the growth of microorganisms. The lowest concentration of the synthesized compounds inhibiting the growth of the given bacteria/fungus was considered as minimal inhibitory concentration (MIC) of the test compounds against that bacteria or fungi on the plate. The MIC values of each compound against various bacteria and fungi were tabulated in Table 2.

RESULTS AND DISCUSSION

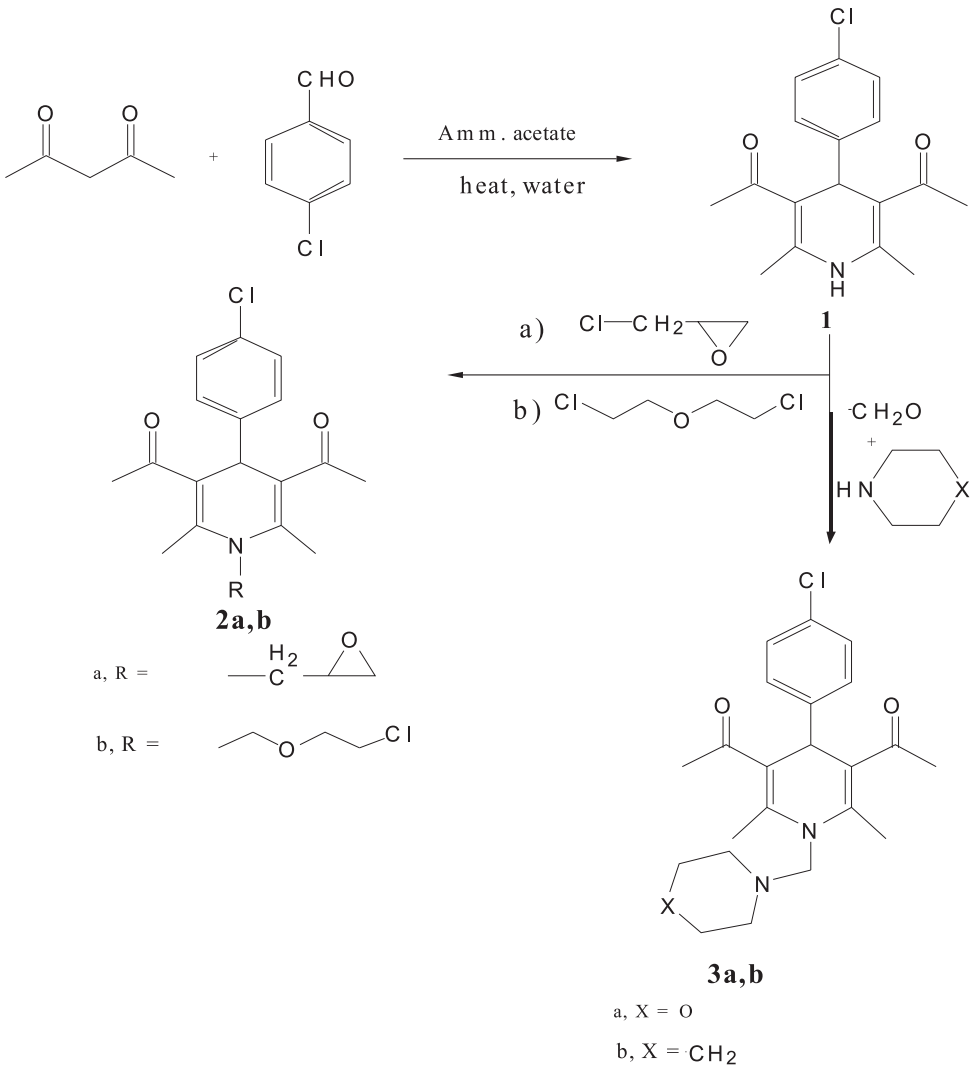
Chemistry

4-(4-Chlorophenyl)-2,6-dimethyl-3,5-bisdiacetyl-1,4-dihydropyridine (**1**) was prepared *via* condensation of 4-chlorobenzaldehyde and acetylacetone in the presence of ammonium acetate. The assignment of the structure was proved based on ele-

Table 1. Inhibition zone in mm as a criterion of antibacterial and antifungal activities of the newly synthesized compounds.

Compound	Microorganism inhibition zone diameter (mm)					
	Gram positive bacteria		Gram negative bacteria		Fungi	
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus niger*
1	14	13	15	14	13	11
2a	13	12	14	13	14	12
3a	17	16	18	17	16	14
3b	23	21	24	22	22	19
8	19	18	20	18	19	15
9	17	17	18	18	18	16
10	17	16	17	17	15	13
12a	25	23	25	25	21	19
Ciprofloxacin	22	24	24	23	-	-
Fluconazole	-	-	-	-	22	24

Highly active = inhibition zone > 20 mm, moderately active = inhibition zone 15-20 mm, slightly active = inhibition zone 11-14 mm, inactive = inhibition zone < 11 mm.



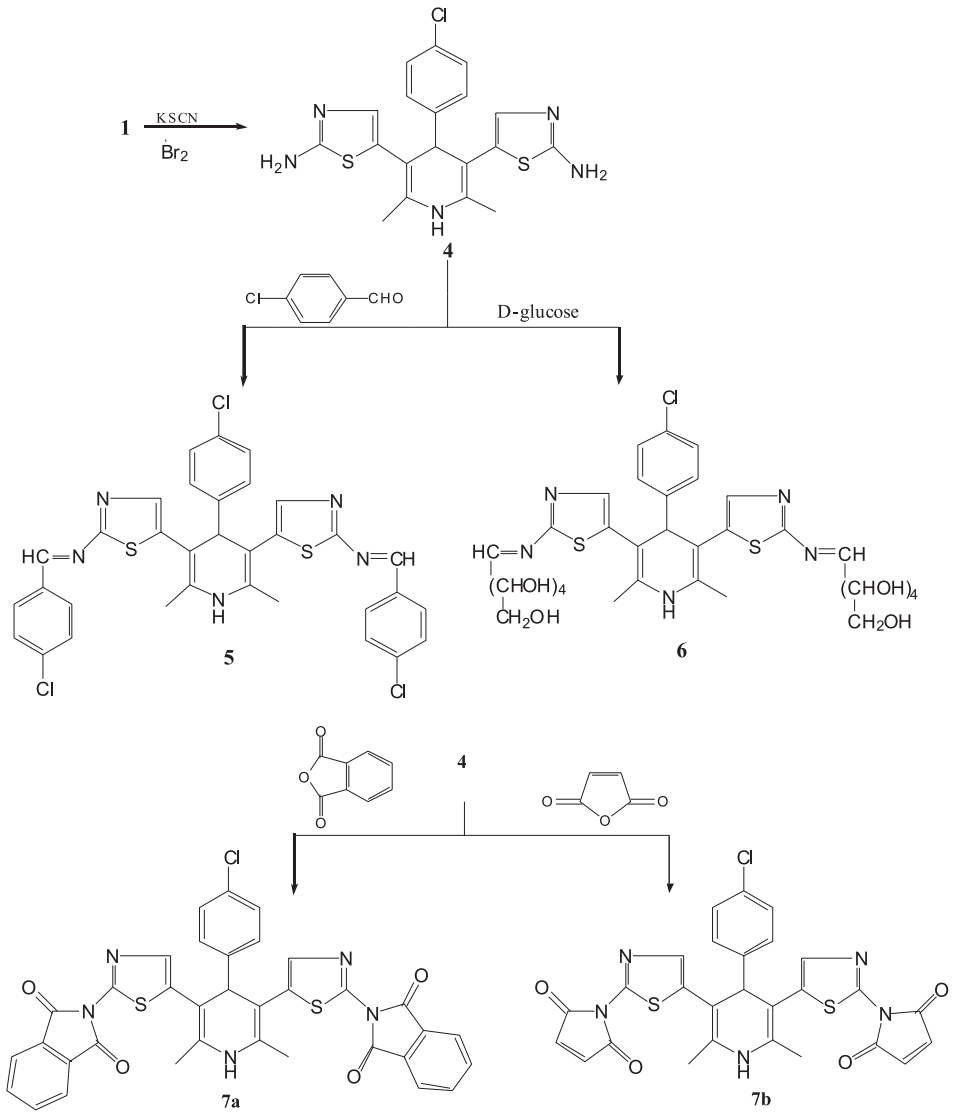
Scheme 1. Synthesis of compounds **1**-**3**

Table 2. MIC in µg/mL of the newly synthesized compounds against microorganisms.

Compound	Gram positive bacteria		Gram negative bacteria		Fungi	
	Bacillus subtilis	*Staphylococcus aureus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Candida albicans*	*Aspergillus niger*
1	1.4	1.6	1.2	1.4	1.6	2
2a	1.8	1.8	1.4	1.6	1.4	1.8
3a	0.8	1	0.6	0.8	1	1.4
3b	0.14	0.18	0.12	0.16	0.16	0.4
8	0.4	0.6	0.2	0.6	0.4	1.2
9	0.8	0.8	0.6	0.6	0.6	1
10	0.8	1	0.8	0.8	1.2	1.6
12a	0.1	0.14	0.1	0.1	0.18	0.4

mental analysis and spectral data. The IR spectrum showed characteristic absorption bands at 1700, 1703 cm^{-1} (2C=O). The ^{1}H-NMR spectrum showed signals at 1.70 ($2CH_3$), 2.30 ($2CH_3$, acetyl), 4.43 (pyridine proton), 7.00-7.15 (Ar-H) and D_2O exchangeable signal at 9.70 ppm assigned for NH. The mass spectrum of **1** showed the molecular ion peak at m/z 303 [M^+, 100], also peak at m/z 305 [M^{2+}, 33] was observed. Compound **1** was transformed chemically *via* the reaction with acyclic alkyl halides yielding N-acyclic nucleoside of pyridine derivatives **2a**,**b**. Mannich adducts also were produced *via* the reaction of **1** with formaldehyde followed by the addition of different amines, namely: morpholine and piperidine affording **3a**,**b**, respectively (Scheme 1). The IR spectra showed no NH absorption for compounds **2** and **3**. The ^{1}H-NMR spectrum of **3b** as representative example showed signals at 1.17-1.49 (m, 6H, piperidine-H), 2.20-2.45 (m, 4H, piperidine-H) and 3.72 ppm (s, 2H, N-CH_2-N). The mass spectrum showed molecular ion peak at m/z 400 (80%).

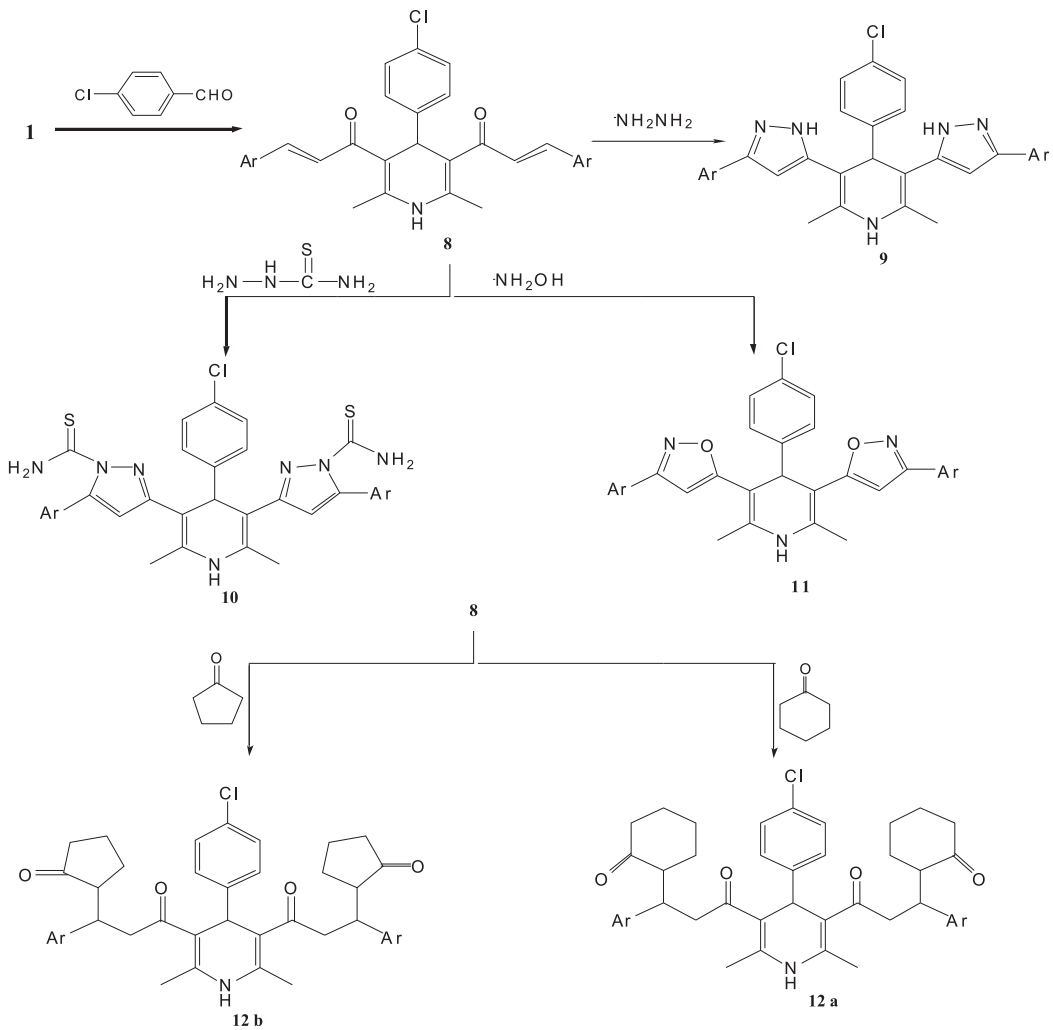
When compound **1** was reacted with potassium thiocyanate in the presence of bromine, 3-bisaminothiazole derivative **4** was produced. The IR spectrum of **4** showed absorption band at 3350 cm^{-1} (NH_2). The ^{1}H-NMR spectrum showed two characteristic signals at 4.00 (NH_2, D_2O exchangeable) and



Scheme 2. Synthesis of compounds **4**-**7**

at 7.50 ppm (thiazole protons). Compound **4** was transformed *via* condensation with 4-chlorobenzaldehyde in glacial acetic acid, glucose in ethanol containing drops of acetic acid and phthalic or maleic anhydrides in glacial acetic acid yielding compounds **5**, **6** and **7a**,**b**, respectively (Scheme 2). The structures of the aforementioned compounds were confirmed on the basis of microanalytical and spectral data. The ^{1}H-NMR spectrum of compound **5** showed a new singlet at 8.10 ppm due to –CH=N-. The mass spectrum of **5** showed a molecular ion peak at m/z 660 supporting its molecular formula. The IR spectrum of **6** was characterized by the appearance of a broad absorption bands of OH and NH groups at the range of 3451–3219 cm^{-1}, while the CH=N appeared at 1590 cm^{-1}. The ^{1}H-NMR spectrum of compound **6** showed the glucose protons as multiplet at the range 3.32–3.92 ppm and the OH groups at the range 4.19–5.00 ppm. The IR spectra of **7** showed bands at 1702–1698 cm^{-1} (C=O). The ^{1}H-NMR spectrum of compound **7b** revealed the presence of two doublets at 6.12 and 6.32 ppm assigned for vinylic protons.

On the other hand, condensation of compound **1** with 4-chlorobenzaldehyde gave bis arylmethylene derivative **8**. The ^{1}H-NMR spectrum of **8** showed absence of $2CH_3$ (acetyl) signals and presence of CH=CH (methylene) at 7.33 and 7.96 ppm. The mass spectrum showed molecular ion peak at m/z 548 (85%). Furthermore, condensation of **8** with different nucleophiles, namely: hydrazine hydrate, thiosemicarbazide and hydroxylamine *via* Micheal condensation reaction gave compounds **9–11,** respectively (Scheme 3).



Scheme 3. Synthesis of compounds **8**-**12**

The structures of compounds **9–11** were in agreement with their spectral and analytical data. The mass spectrum of compound **9** showed a molecular ion peak at m/z 572 (55%). Its ^{1}H-NMR spectrum showed singlet at 6.50 ppm characteristic for pyrazole ring protons. Compound **8**, when condensed with cyclohexanone and cyclopentanone, afforded compounds **12a**,**b**. The IR spectrum of **12a** showed absorption bands at1695, 1698, 1700–1705, 1710 cm^{-1} (C=O). The ^{1}H-NMR spectrum of **12a** showed multiplet at 1.80–2.59 for 18 protons of cyclohexanone, signals at 3.17–3.20 for 2CH-propyl protons and at 3.25–3.40 ppm for 2CH_2-propyl protons. The mass spectrum of **12a** showed molecular ion peak at m/z 745 (100%).

Antimicrobial activity

All the newly synthesized compounds were screened for their *in vitro* antibacterial activity against two strains of Gram positive bacteria *(Staphylococcus aureus, Bacillus subtilis*), and two strains of Gram negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*) using ciprofloxacin as a standard drug (100 μg/mL). They were also evaluated for their *in vitro* antifungal activity against the mycotic strains (*Candida albicans* and *Aspergillus niger*) using fluconazole as a standard antifungal drug (100 μg/mL). Agar-diffusion method was used in this investigation for determination of the preliminary antibacterial and antifungal activity and the results were recorded for each tested compound as the average diameter of inhibition zones (IZ) of bacterial or fungal growth around the discs in mm (Table 1). The minimal inhibitory concentrations (MIC) were determined for compounds showing promising growth inhibition, using the twofold serial dilution method (43). The MIC (μg/mL) values against the tested bacterial and fungal isolates are presented in Table 2.

According to Tables 1 and 2, it is clear that compounds **1**, **2a** and **3a** showed low activities toward all types of microorganisms. Compounds **9** and **10** showed moderate antibacterial and antifungal activities. Bis-arylmethylene derivative **8** was found to be highly active against *Escherichia coli*, but showed moderate activity towards Gram positive bacteria, Gram negative bacteria and fungi. Derivatives **3b**, 3,5-bis-pyridin-1H-morpholine and **12a**, dihydropyridine cyclohexanone, showed high activity toward all microorganisms.

CONCLUSION

In the present study, 2,6-dimethyl-3,5-bis-acetyl-1,4-dihydropyridine (**1**) was used to synthesize novel derivatives of N-acyclic nucleosides (**2a**,**b**), Mannich products (**3a**,**b**), 3,5-bis-aminothiazole (**4**), heterocyclic derivatives (**5**-**7**), 3,5-bisarylmethylene (**8**) and (**9**-**12**). The antimicrobial activity of some compounds was reported. Compounds **3b** and **12a** showed high activity against Gram positive bacteria, Gram negative bacteria and fungi.

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SYNTHESIS AND ANTIMICROBIAL EVALUATION OF CYANOPYRIDINYL TETRAHYDRONAPHTHALENE DERIVATIVES

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Abstract: A novel series of cyanopyridinyl tetrahydronaphthalene incorporated with different heterocycles were synthesized. The key compounds **2a**,**b** were condensed with chloroacetone and ethyl chloroacetate to give **3a**,**b** and **4a**,**b**, respectively. Also condensation of **4a**,**b** with hydrazine hydrate gave the corresponding hydrazide **5a**,**b**. Reaction of **5b** with different isothiocyanates gave the corresponding thiosemicarbazide derivatives **6a-c**. Also, condensation of **5a** with chloroacetic acid, methyl iodide and /or acetic anhydride yielded **7-9**, respectively. Moreover, reaction of **5a** with acetylacetone, ethyl acetoacetate, diethylmalonate, ethyl cyanoacetate, chloroacetone, ethyl chloroacetate, urea, phthalic anhydride, malic anhydride and/ or different aldehydes yielded the corresponding derivatives **10-18**, respectively. Newly synthesized compounds were screened for their antibacterial (*Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Escherichia* coli) and antifungal (*Saccharomyces cerevisiae and Candida albicans*) activity. The results revealed that some of novel compounds have exhibited significant biological activity against the tested microorganisms.

Keywords: tetrahydronaphthalene, cyanopyridine, antimicrobial activity

In the last twenty five years there has been a steep decline in the commercial output and research and development of antimicrobial agents by the major pharmaceutical companies due to the more attractive commercial returns that can be made for treatments of chronic human diseases. At the same time, there has been an explosion both in the numbers of pathogenic bacteria that have become resistant to antibiotics due to their widespread and misuse and of immuno-compromised patients that are particularly susceptible to opportunistic pathogens (1, 2). In addition, it is known that antifungal drugs do not have selective activity because of the biochemical similarity between human cell and fungi forms (3). Therefore, the discovery of new antimicrobial agents with novel modes of action and no cross-resistance with current antibiotics will be vital to meet the threats created by the emergence of bacteria resistant to the current therapeutic agents.

Literature survey revealed that tetralin is an important ring comprising different efficacious antimicrobial derivatives (4, 5). Additionally, the pyridine nucleus plays a key role of catalyzing both biological and chemical systems. In many enzymes of living organisms it is the prosthetic pyridine nucleotide (NADP) that is involved in various oxidation–reduction processes. It is also one of the most important heterocycles found in many antimicrobial pharmaceuticals (6, 7).

Since it is documented that the most effective antimicrobial compounds can be designed by joining two or more biologically active cyclic systems together in a single molecular framework, we report here the synthesis and antimicrobial evaluation of some novel structure hybrids incorporating both the tetralin moiety with pyridine heterocycle. This combination was suggested in an attempt to investigate the influence of such hybridization and structure variation on the anticipated biological activities,

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hoping to add some synergistic biological significance to the target molecules.

EXPERIMENTAL

All melting points are uncorrected and were taken in open capillary tubes using Electrothermal apparatus 9100. Elemental microanalyses were carried out at the Microanalytical Unit, Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt, and were found to be within ± 0.5% of the theoretical values. Infrared spectra were recorded on a Jasco FT/IR-6100 Fourier transform infrared spectrometer using the KBr disc technique at the Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt. ^{1}H NMR spectra were determined by using a Jeol EX-270 NMR spectrometer at the Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt. The mass spectra were measured with a Finnigan MAT SSQ-7000 mass spectrometer at Central Services Laboratory, National Research Centre, Dokki, Cairo, Egypt. Follow-up of the reactions and checking of the purity of the compounds were made by TLC on silica gel precoated aluminum sheets (Type 60, F 254, Merck, Darmstadt, Germany) and the spots were detected by exposure to a UV lamp at 254 nm for a few seconds. The chemical names for the prepared compounds are given according to the IUPAC system.

General procedure for the synthesis of 1,2-dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-substituted-2-oxopyridine-3-carbonitrile 1a,**b**

A mixture of 6-acetyl-1,2,3,4-tetrahydronaphthalene (6.0 g, 0.034 mol), the appropriate aldehyde namely: 3,4-dimethoxybenzaldehyde and/or 4-fluorobenzaldehyde (0.034 mol), ethyl cyanoacetate (3.8 mL, 0.034 mol) and ammonium acetate (21.0 g, 0.272 mol) in n-butanol (50 mL) was refluxed for 6 h. The formed precipitate was filtered, washed with ether, dried and recrystallized from acetic acid to give the title compounds **1a**,**b**, respectively.

1,2-Dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)-2-oxopyridine-3-carbonitrile (1a)

Yield 65%; m.p. 250-252^{o}C; IR (KBr, cm^{-1}): 2930, 2850 (CH_2 - tetrahydronaphthalene protons), 2219 (CN), 1637 (CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.74, 2.68 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.83 (6H, s, 2-OCH_3), 6.98-7.23 (7H, m, Ar-H and pyridone proton), 9.21 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 386 [M$^+$] (100); Analysis: calcd. for $C_{24}H_{22}N_2O_3$ (386.44): C, 74.59; H, 5.74; N, 7.25%; found: C, 74.89; H, 5.94; N, 7.35%.

4-(4-Fluorophenyl)-1,2-dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-oxopyridine-3-carbonitrile (8)

General procedure for the synthesis of 6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-mercapto-4-substituted-pyridine-3-carbonitrile 2a,b

A mixture of compounds **1a**,**b** (0.01 mol) and P_2S_5 (2.22 g, 0.01 mol) in pyridine (15 mL) was refluxed for 5 h. After cooling, the mixture was poured onto ice/cold water and acidified with hydrochloric acid. The formed precipitate was filtered, washed with water several times, dried and recrystallized from ethanol to give the title compounds **2a**,**b**, respectively.

6-(1,2,3,4-Tetrahydronaphthalen-6-yl)-2-mercapto-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (2a)

Yield 65%; m.p. 107-108^{o}C; IR (KBr, cm^{-1}): 2925, 2852 (CH_2 - tetrahydronaphthalene protons), 2215 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.72, 2.74 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.81 (6H, s, 2-OCH_3), 6.98-7.23 (7H, m, Ar-H and pyridine proton), 8.81 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 402 [M$^+$] (100); Analysis: calcd. for $C_{24}H_{22}N_2O_2S$ (402.51): C, 71.62; H, 5.51; N, 6.96; S, 7.79%; found: C, 71.32; H, 5.01; N, 6.76; S, 7.54%.

4-(4-Fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-mercaptopyridine-3-carbonitrile (2b)

Yield 55%; m.p. 95-96^{o}C; IR (KBr, cm^{-1}): 2930, 2850 (CH_2 - tetrahydronaphthalene protons), 2219 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.73, 2.69 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 6.81-7.54 (8H, m, Ar-H and pyridine proton), 8.75 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 360 [M$^+$] (100); Analysis: calcd. for $C_{22}H_{17}FN_2S$ (360.45): C, 73.31; H, 4.75; N, 7.77; S, 8.90%; found: C, 73.41; H, 4.82; N, 7.93; S, 8.75%.

General procedure for the synthesis of 2-(2-oxopropylthio)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-substituted-pyridine-3-carbonitrile 3a,b and ethyl 2-(3-cyano-6-(1,2,3,4-tetrahydronaphhalen-6-yl)-4-substituted-pyridin-2-ylthio)acetate 4a,**b**

A mixture of compounds **2a**,**b** (0.003 mol), anhydrous sodium carbonate (0.32 g, 0.003 mol)

and the appropriate halo derivatives, namely: chloroacetone and/or ethyl chloroacetate (0.003 mol) in DMF (20 mL) was refluxed for 4 h. The reaction mixture was cooled and poured onto ice/cold water and acidified with hydrochloric acid. The formed precipitate was filtered, washed with water several times, dried and recrystallized from methanol to give the title compounds **3a**,**b** and **4a**,**b**, respectively.

2-(2-Oxopropylthio)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (3a)

Yield 35%; m.p. 149-150°C; IR (KBr, cm^{-1}): 2929, 2853 (CH_2 - tetrahydronaphthalene protons), 2215 (CN), 1702 (CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70, 2.71 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.28 (3H, s, CH_3CO), 3.83 (6H, s, 2-OCH_3), 4.10 (2H, s, SCH_2), 6.72-7.91 (7H, m, Ar-H and pyridine proton); MS, m/z (%): 458 [M^+] (70), 459 [M^++1] (38), 73 [C_2H_3NS] (100); Analysis: calcd. for $C_{27}H_{26}N_2O_3S$ (458.57): C, 70.72; H, 5.71; N, 6.11; S, 6.99%; found: C, 70.43; H, 5.91; N, 6.61; S, 7.13%.

2-(2-Oxopropylthio)-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridine-3-carbonitrile (3b)

Yield 40%; m.p. 114-115°C; IR (KBr, cm^{-1}): 2929, 2857 (CH_2 - tetrahydronaphthalene protons), 2216 (CN), 1709 (CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.69, 2.68 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.17 (3H, s, CH_3CO), 3.79 (6H, s, 2-OCH_3), 4.12 (2H, s, SCH_2), 6.83-7.85 (8H, m, Ar-H and pyridine proton); MS, m/z (%): 416 [M^+] (100); Analysis: calcd. for $C_{25}H_{21}FN_2OS$ (416.51): C, 72.09; H, 5.08; N, 6.73; S, 7.70%; found: C, 72.31; H, 4.98; N, 6.82; S, 7.56%.

Ethyl 2-(3-cyano-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridin-2-ylthio)acetate (4a)

Yield 70%; m.p. 164-165°C; IR (KBr, cm^{-1}): 2931, 2834 (CH_2 - tetrahydronaphthalene protons), 2214 (CN), 1725 (CO, ester); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.29 (3H, t, -$COOCH_2\underline{CH_3}$), 1.69, 2.72 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.75 (6H, s, 2-OCH_3), 4.13 (2H, s, SCH_2), 4.16 (2H, q, -$COO\underline{CH_2}CH_3$), 7.05-7.99 (7H, m, Ar-H and pyridine proton); MS, m/z (%): 488 [M^+] (35), 115 (C_9H_7) (100); Analysis: calcd. for $C_{28}H_{28}N_2O_4S$ (488.60): C, 68.83; H, 5.78; N, 5.73; S, 6.56%; found: C, 68.83; H, 5.91; N, 5.63; S, 6.68%.

Ethyl 2-(3-cyano-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridin-2-ylthio)acetate (4b)

Yield 68%; m.p. 131-132°C; IR (KBr, cm^{-1}): 2921, 2830 (CH_2 - tetrahydronaphthalene protons), 2217 (CN), 1740 (CO, ester); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.24 (3H, t, -$COOCH_2\underline{CH_3}$), 1.71, 2.67 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.78 (6H, s, 2-OCH_3), 4.11 (2H, s, SCH_2), 4.15 (2H, q, -$COO\underline{CH_2}CH_3$), 7.05-7.89 (8H, m, Ar-H and pyridine proton);; MS, m/z (%): 446 [M^+] (15), 357 [$C_{22}H_{14}FN_2S$] (100); Analysis: calcd. for $C_{26}H_{23}FN_2O_2S$ (446.54): C, 69.93; H, 5.19; N, 6.27; S, 7.18%; found: C, 70.28; H, 5.44; N, 6.35; S, 7.23%.

General procedure for the synthesis of 2-hydrazinyl-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(substituted phenyl)pyridine-3-carbonitrile 5a,b

Method A

A mixture of compound **2a**,**b** (0.01 mol) and hydrazine hydrate (1.0 mL, 0.02 mol) in absolute ethanol was refluxed for 4 h. After cooling the formed precipitate was filtered, dried and recrystallized from methanol to give the title compound **5a**,**b**.

Method B

A mixture of compounds **4a**,**b** (0.002 mol) and hydrazine hydrate (0.1 mL, 0.002 mol) in absolute ethanol (20 mL) was refluxed for 3 h. The formed precipitate was filtered, dried and recrystallized from dioxane to give the title compounds **5a**,**b**, respectively.

2-Hydrazinyl-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (5a)

Yield 88%; m.p. 139-140°C; IR (KBr, cm^{-1}): 3245, 3177 (NH, NH_2), 2935, 2855 (CH_2 - tetrahydronaphthalene protons), 2216 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.67, 2.68 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.78 (6H, s, 2-OCH_3), 6.95-7.98 (7H, m, Ar-H and pyridine proton), 8.98, 9.01 (3H, s, $NHNH_2$, exchangeable by D_2O); MS, m/z (%): 400 [M^+] (32), 402 [M^+ + 2] (65), 370 [M^+-N_2H_2] (100); Analysis: calcd. for $C_{24}H_{24}N_4O_2$ (400.47): C, 71.98; H, 6.04; N, 13.99%; found: C, 71.74; H, 6.28; N, 13.68%.

4-(4-Fluorophenyl)-2-hydrazinyl-6-(1,2,3,4-tetrahydronaphthalen -6-yl)pyridine-3-carbonitrile (5b)

Yield 63%; m.p. 128-129°C; IR (KBr, cm^{-1}): 3336, 3208 (NH, NH_2), 2932, 2850 (CH_2 - tetrahy-

dronaphthalene protons), 2217 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.69, 2.66 (8H, m, 4(CH_2) – tetrahydronaphthalene protons), 7.04-8.04 (8H, m, Ar-H and pyridine proton), 8.67, 9.22 (3H, s, $NHNH_2$, exchangeable by D_2O); MS, m/z (%): 358 [M^+] (95), 359 [M^+ + 1] (30), 77 [C_6H_5] (100); Analysis: calcd. for $C_{22}H_{19}FN_4$ (358.41): C, 73.72; H, 5.34; N, 15.63%; found: C, 73.51; H, 5.21; N, 15.78%.

General procedure for the synthesis of 1-(3-cyano-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridin-2-yl)-4- substituted thiosemicarbazide 6a-c

A mixture of compound **5b** (0.87 g, 0.002 mol), the appropriate isothiocyanate, namely: methylisothiocyanate, ethylisothiocyanate and/or phenylisothiocyanate in dry benzene (20 mL) was refluxed for 6 h. The solvent was evaporated under reduced pressure and the remaining solid was recrystallized from methanol to give the title compounds **6a-c**, respectively.

1-(3-Cyano-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridin-2-yl)-4-methylthiosemicarbazide (6a)

Yield 50%; m.p. 158-159°C; IR (KBr, cm^{-1}): 3372, 3256, 3151 (3NH), 2929, 2845 (CH_2 - tetrahydronaphthalene protons), 2216 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71, 2.82 (8H, m, 4(CH_2)-tetrahydronaphthalene protons), 2.56 (3H, s, -$NHCH_3$), 7.03-7.95 (8H, m, Ar-H and pyridine proton), 8.50, 8.68, 9.01 (3H, s, 3NH, exchangeable by D_2O); MS, m/z (%): 399 [M^+ - CH_6N] (30), 358 [$C_{22}H_{19}FN_4$] (100); Analysis: calcd. for $C_{24}H_{22}FN_5S$ (431.53): C, 66.80; H, 5.14; N, 16.23; S, 7.43%; found: C, 66.58; H, 5.29; N, 16.51; S, 7.57%.

1-(3-Cyano-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridin-2-yl)-4-ethylthiosemicarbazide (6b)

Yield 45%; m.p. 215-216°C; IR (KBr, cm^{-1}): 3370, 3251, 3166 (3NH), 2932, 2851 (CH_2 - tetrahydronaphthalene protons), 2214 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.05 (3H, t, -$NHCH_2\underline{CH_3}$), 1.69, 2.78 (8H, m, 4(CH_2) – tetrahydronaphthalene protons), 3.51 (3H, q, -$NH\underline{CH_2}CH_3$), 7.07-8.10 (8H, m, Ar-H and pyridine proton), 8.41, 8.72, 9.21 (3H, s, 3NH, exchangeable by D_2O); MS, m/z (%): 418 [M^+ - C_2H_3] (6), 401 [M^+ - C_2H_5N] (100); Analysis: calcd. for $C_{25}H_{24}FN_5S$ (445.55): C, 67.39; H, 5.43; N, 15.72; S, 7.20%; found: C, 67.53; H, 5.34; N, 15.60; S, 7.44%.

1-(3-Cyano-4-(4-fluorophenyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)pyridin-2-yl)-4-phenylthiosemicarbazide (6c)

Yield 39%; m.p. 169-170°C; IR (KBr, cm^{-1}): 3362, 3279, 3181 (3NH), 2931, 2855 (CH_2 - tetrahydronaphthalene protons), 2218 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71, 2.73 (8H, m, 4(CH_2) – tetrahydronaphthalene protons), 7.12-7.97 (13H, m, Ar-H and pyridine proton), 8.38, 8.64, 8.89 (3H, s, 3NH, exchangeable by D_2O); MS, m/z (%): 399 [M^+ - C_6H_8N] (4), 77 [C_6H_5] (100); Analysis: calcd. for $C_{29}H_{24}FN_5S$ (493.60): C, 70.57; H, 4.90; N, 14.19; S, 6.50%; found: C, 70.29; H, 4.58; N, 14.31; S, 6.82%.

2-(2-Hydrazinyl-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carboyl)acetic acid (7)

A mixture of compound **5a** (2.0 g, 0.005 mol), chloroacetic acid (0.47 g, 0.005 mol) and anhydrous sodium acetate (0.82 g, 0.01 mol) in acetic anhydride (25 mL) and glacial acetic acid (50 mL) was refluxed for 6 h. After cooling, the reaction mixture was poured onto ice/cold water; the formed precipitate was filtered, dried, and recrystallized from ethanol to give compound **7**.

Yield 58%; m.p. 129-130°C; IR (KBr, cm^{-1}): 3417 (COOH), 2925, 2853 (CH_2 - tetrahydronaphthalene protons), 2214 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.68, 2.69 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.51 (2H, s, -CH_2), 3.76 (6H, s, 2-OCH_3), 6.88-8.01 (7H, m, Ar-H and pyridine proton), 8.23, 8.64, 11.21 (3H, s, 2NH, OH, exchangeable by D_2O); MS, m/z (%): 460 [M^+ + 2] (11), 441 [M^+ - OH] (38), 386 [M^+ - $C_2H_2NO_2$] (100); Analysis: calcd. for $C_{26}H_{26}N_4O_4$ (458.51): C, 68.11; H, 5.72; N, 12.22%; found: C, 68.24; H, 5.61; N, 12.43%.

2-(2-Methylhydrazinyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (8)

A mixture of compound **5a** (0.8 g, 0.002 mol), anhydrous sodium carbonate (0.21 g, 0.002 mol) and iodomethane (0.29 mL, 0.002 mol) in DMF (20 mL) was refluxed for 7 h. Then, the reaction mixture was cooled, poured onto ice/cold water. The formed precipitate was filtered, dried, and recrystallized from acetic acid to give the title compound **8**.

Yield 76%; m.p. 114-115°C; IR (KBr, cm^{-1}): 3352, 3215 (2NH), 2921, 2853 (CH_2 - tetrahydronaphthalene protons), 2216 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.69, 2.72 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.56 (2H, s, -CH_3), 3.76 (6H, s, 2-OCH_3), 6.75-8.12 (7H, m, Ar-H and pyri-

dine proton), 8.11, 8.53 (2H, s, 2NH, exchangeable by D_2O); MS, m/z (%): 414 [M^+] (26), 416 [$M^+ + 2$] (76), 77 [C_6H_5] (100); Analysis: calcd. for $C_{25}H_{26}N_4O_2$ (414.50): C, 72.44; H, 6.32; N, 13.52%; found: C, 72.28; H, 6.52; N, 13.33%.

N'-(3-cyano-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridin-2-yl)acetohydrazide (9)

A mixture of compound **5a** (0.40 g, 0.001 mol) and acetic anhydride (0.11 mL, 0.001 mol) in acetic acid (10 mL) was refluxed for 6 h. The reaction mixture was cooled and poured onto ice/cold water. The formed precipitate was filtered, dried and recrystallized to give the title compound **9**.

Yield 65%; m.p. 101-103°C; IR (KBr, cm^{-1}): 3425, 3258 (2NH), 2930, 2857 (CH_2 - tetrahydronaphthalene protons), 2216 (CN), 1633 (CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.68, 2.59 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.12 (3H, s, CH_3), 3.73 (6H, s, 2-OCH_3), 6.88-7.74 (7H, m, Ar-H and pyridine proton), 7.91, 8.25 (2H, s, 2NH, exchangeable by D_2O); MS, m/z (%): 442 [M^+] (8), 415 [$C_{25}H_{25}N_3O_3$] (100); Analysis: calcd. for $C_{26}H_{26}N_4O_3$ (442.51): C, 70.57; H, 5.92; N, 12.66%; found: C, 70.41; H, 5.71; N, 12.43%.

6-(1,2,3,4-Tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)-2-(3,5-dimethyl-1H-pyrazol-1-yl)pyridine-3-carbonitrile (10)

Refluxing a mixture of compound **5a** (0.40 g, 0.001 mol) and acetylacetone (0.10 mL, 0.001 mol) in acetic acid (15 mL) for 6 h. The formed precipitate after cooling was filtered, dried and recrystallized from acetic acid to give the title compound **10**. Yield 52%: m.p. 114-115°C; IR (KBr, cm^{-1}): 2928, 2855 (CH_2 - tetrahydronaphthalene protons), 2214 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.69, 2.71 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.94 (6H, s, 2CH_3-pyrazole ring), 3.78 (6H, s, 2-OCH_3), 6.01 (1H, s, pyrazole proton), 6.82-8.21 (7H, m, Ar-H and pyridine proton); MS, m/z (%): 414 [M^+ - C_4H_2] (42), 344 [M^+ - $C_6H_6N_3$] (50), 55 [C_4H_7] (100); Analysis: calcd. for $C_{29}H_{28}N_4O_2$ (464.56): C, 74.98; H, 6.08; N, 12.06%; found: C, 74.73; H, 5.94; N, 12.26%.

General procedure for the synthesis of compounds (11-13)

A mixture of compound **5a** (0.40 g, 0.001 mol) and active methylene compounds, namely: ethyl acetoacetate, diethylmalonate and/or ethyl cyanoacetate (0.001 mol) in ethanol (20 mL) containing few drops of piperidine was refluxed for 4 h. The formed precipitate was filtered, dried and recrystallized from methanol to give the title compounds **11-13**, respectively.

2-(4,5-Dihydro-3-methyl-5-oxopyrazol-1-yl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (11)

Yield 60%; m.p. 120-121°C; IR (KBr, cm^{-1}): 2928, 2857 (CH_2 - tetrahydronaphthalene protons), 2211 (CN), 1635 (CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.69, 2.71 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.02 (3H, s, CH_3), 2.21 (2H, s, CH_2 - pyrazole proton), 3.79 (6H, s, 2-OCH_3), 6.80-8.32 (7H, m, Ar-H and pyridine proton); MS, m/z (%): 452 [M^+ - CH_2] (2), 402 [$C_{24}H_{26}N_4O_2$] (100); Analysis: calcd. for $C_{28}H_{26}N_4O_3$ (466.53): C, 72.09; H, 5.62; N, 12.01%; found: C, 72.35; H, 5.81; N, 12.27%.

6-(1,2,3,4-Tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)-2-(3,5-dioxopyrazolidin-1-yl) pyridine-3-carbonitrile (12)

Yield 75%; m.p. 107-108°C; IR (KBr, cm^{-1}): 3428 (NH), 2927, 2855 (CH_2 - tetrahydronaphthalene protons), 2215 (CN); 1735, 1633 (CO cyclic); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.72, 2.75 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.11 (2H, s, CH_2 - pyrazole proton), 3.79 (6H, s, 2-OCH_3), 6.95-8.42 (7H, m, Ar-H and pyridine proton), 8.69 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 443 [$C_{26}H_{25}N_3O_2$] (3), 56 [C_4H_8] (100); Analysis: calcd. for $C_{27}H_{24}N_4O_4$ (468.50): C, 69.22; H, 5.16; N, 11.96%; found: C, 69.39; H, 5.41; N, 11.75%.

2-(3-Amino-4,5-dihydro-5-oxopyrazol-1-yl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (13)

Yield 80%; m.p. 145-146°C; IR (KBr, cm^{-1}): 3386, 3124 (NH_2), 2928, 2855 (CH_2 - tetrahydronaphthalene protons), 2210 (CN), 1736 (CO, cyclic); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.72, 2.74 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.20 (2H, s, CH_2), 3.78 (6H, s, 2-OCH_3), 6.95-7.98 (7H, m, Ar-H and pyridine proton), 9.98 (2H, s, NH_2, exchangeable by D_2O); MS, m/z (%): 467 [M^+] (8), 402 [$C_{24}H_{26}N_4O_2$] (100); Analysis: calcd. for $C_{27}H_{25}N_5O_3$ (467.52): C, 69.36; H, 5.39; N, 14.98%; found: C, 69.61; H, 5.60; N, 14.77%.

3,4-Dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-8-(3,4-dimethoxyphenyl)-3-methyl-2H-pyrido[2,1-c][1,2,4]triazine-9-carbonitrile (14)

A mixture of compound **5a** (0.40 g, 0.001 mol) and chloroacetone (0.10 mL, 0.001 mol) in dry

Table 1. Antimicrobial activity expressed as inhibition diameter zones in millimeters (mm) of chemical compounds against the pathological strains based on well diffusion assay.

Compound No.	Gram positive bacteria				Gram negative bacteria			Yeast	
	Staphylococcus aureus ATCC 29213	*Bacillus subtilis* ATCC6633	*Bacillus megaterium* ATCC 9885	*Sarcina lutea*	*Klebsiella pneumoniae* ATCC13883	*Pseudomonas aeruginosa* ATCC27953	*Escherichia coli* ATCC25922	*Saccharomyces cerevisiae*	*Candida albicans* NRRL Y-477
1a	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	21	20
2a	20	22	20	21	26	20	22	23	22
2b	27	24	29	20	26	28	25	33	32
3a	17	17	17	19	N.A.	N.A.	N.A.	N.A.	18
3b	20	20	18	22	22	18	20	21	25
4a	17	20	16	17	17	18	16	21	26
4b	17	N.A.	N.A.	16	16	16	17	18	20
5a	30	35	28	34	33	35	32	24	28
5b	17	25	N.A.	N.A.	30	28	32	29	25
6a	25	22	28	22	16	18	17	16	16
6b	15	26	28	29	24	20	20	29	23
6c	18	20	15	16	19	19	20	18	22
7	19	18	16	19	21	20	20	18	20
8	13	18	14	15	25	23	22	24	22
9	19	12	16	20	15	14	13	18	20
10	19	14	17	N.A.	18	17	17	23	22
11	17	15	16	17	19	18	16	20	20
12	17	17	16	16	17	18	17	25	21
13	15	17	14	88	19	19	18	20	18
14	17	16	15	18	15	13	14	21	22
15	18	15	15	19	N.A.	16	17	22	20
16	18	13	16	18	14	18	13	25	21

Table 1. cont.

Compound No.	Gram positive bacteria				Gram negative bacteria			Yeast	
	Staphylococcus aureus ATCC 29213	*Bacillus subtilis* ATCC6633	*Bacillus megaterium* ATCC 9885	*Sarcina lutea*	*Klebsiella pneumoniae* ATCC13883	*Pseudomonas aeruginosa* ATCC27953	*Escherichia coli* ATCC25922	*Saccharomyces cerevisiae*	*Candida albicans* NRRL Y-477
17a	14	14	15	15	26	25	27	29	26
18a	20	16	15	18	18	18	17	17	N.A.
18b	14	18	16	16	17	15	14	20	18
18c	23	17	13	27	18	18	19	17	18
18d	18.	17	15	16	16	16	17	18	15
Ciprofloxacin	20	22	24	20	25	24	23	N.A.	N.A.
Ketoconazole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	23	22

The experiment was carried out in triplicate and the average zone of inhibition was calculated. N.A. = not active.

xylene (20 mL) was refluxed for 5 h. The formed precipitate that separated while hot was collected and recrystallized from ethanol to give the title compound **14**.

Yield 78%; m.p. 122-123°C; IR (KBr, cm^{-1}): 3423 (NH), 2922, 2852 (CH_2 - tetrahydronaphthalene protons), 2212 (CN); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.13 (3H, d, CH_3), 1.70, 2.67 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.79 (2H, m, CH_2 - triazine protons), 3.34 (1H, m, CH - triazine proton), 3.78 (6H, s, 2-OCH_3), 6.65-7.89 (7H, m, Ar-H and pyridine proton), 8.51 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 443 [M^+ + 3] (8), 57 [C_4H_9] (100); Analysis: calcd. for $C_{27}H_{28}N_4O_2$ (440.54): C, 73.61; H, 6.41; N, 12.72%; found: C, 73.50; H, 6.61; N, 12.51%.

3,4-Dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-8-(3,4-dimethoxyphenyl)-3-oxo-2H-pyrido[2,1-c][1,2,4]triazine-9-carbonitrile (15)

A mixture of compound **5a** (0.40 g, 0.001 mol) and ethyl chloroacetate (0.12 mL, 0.001 mol) in ethanol (20 mL) was refluxed for 6 h. The formed precipitate after cooling was filtered, dried and recrystallized form methanol to give the title compound **15**.

Yield 58%; m.p. 140-141°C; IR (KBr, cm^{-1}): 3448 (NH), 2928, 2852 (CH_2 - tetrahydronaphthalene protons), 2213 (CN), 1740 (CO, cyclic); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.70, 2.67 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.51 (2H, s, CH_2 - triazine protons), 3.79 (6H, s, 2-OCH_3), 6.71-7.92 (7H, m, Ar-H and pyridine proton), 8.77 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 429 [$C_{26}H_{27}N_3O_3$] (2), 59 [C_4H_{11}] (100); Analysis: calcd. for $C_{26}H_{24}N_4O_3$ (440.49): C, 70.89; H, 5.49; N, 12.72%; found: C, 70.62; H, 5.38; N, 12.81%.

2,3-Dihydro-5-(1,2,3,4-tetrahydronaphthalen-6-yl)-7-(3,4-dimethoxyphenyl)-3-oxo-[1,2,4]triazolo[4,3-a]pyridine-8-carbonitrile (16)

A mixture of compound **5a** (0.40 g, 0.001 mol) and urea (0.01mol) was heated at 190-200°C for 6 h. After cooling, the reaction mixture was triturated with hot water, The formed precipitate was filtered, dried and recrystallized from ethanol to give the title compound **16**.

Yield 38%; m.p. 203-204°C; IR (KBr, cm^{-1}): 3204 (NH), 2927, 2854 (CH_2 - tetrahydronaphthalene protons), 2218 (CN), 1693 (CO, cyclic); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71, 2.70 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.83 (6H, s, 2-OCH_3), 6.97-7.91 (6H, m, Ar-H and pyridine proton), 8.64 (1H, s, NH, exchangeable by D_2O);

Table 2 Minimum inhibitory concentration (μg/mL) against the pathological strains based on twofold serial dilution technique.

Compound No.	Gram positive bacteria				Gram negative bacteria			Yeast	
	Staphylococcus aureus ATCC 29213	*Bacillus subtilis* ATCC6633	*Bacillus megaterium* ATCC 9885	*Sarcina lutea*	*Klebsiella pneumoniae* ATCC13883	*Pseudomonas aeruginosa* ATCC27953	*Escherichia coli* ATCC25922	*Saccharomyces cerevisiae*	*Candida albicans* NRRL Y-477
1a	-	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	100	100
2a	100	100	100	100	50	100	50	50	50
2b	50	50	50	50	50	50	50	25	25
3a	200	200	200	200	-	-	-	-	200
3b	20	20	18	22	22	18	20	100	50
4a	200	200	200	200	200	18200	200	100	100
4b	200	-	-	200	200	200	200	200	200
5a	25	25	25	25	25	25	25	50	50
5b	200	50	-	-	50	50	25	50	50
6a	50	100	50	100	200	200	200	200	200
6b	-	50	50	50	100	100	100	50	50
6c	200	200	-	200	200	200	200	200	100
7	200	200	200	200	200	200	200	200	200
8	-	200	-	-	100	100	100	100	100
9	200	-	200	100	-	-	-	200	100
10	200	-	200	-	200	200	200	100	50
11	200	-	200	200	200	200	200	200	200
12	200	200	200	200	200	200	200	50	50
13	-	200	-	200	200	200	200	200	200
14	200	200	-	200	-	-	-	100	100
15	200	-	-	200	-	200	200	100	100
16	200	-	200	200	-	200	-	50	100

Table 2. cont.

Compound No.	Gram positive bacteria				Gram negative bacteria			Yeast	
	Staphylococcus aureus ATCC 29213	*Bacillus subtilis* ATCC6633	*Bacillus megaterium* ATCC 9885	*Sarcina lutea*	*Klebsiella pneumoniae* ATCC13883	*Pseudomonas aeruginosa* ATCC27953	*Escherichia coli* ATCC25922	*Saccharomyces cerevisiae*	*Candida albicans* NRRL Y-477
17a	-	-	-	-	100	100	50	50	100
18a	100	200	-	200	200	200	200	200	N.A.
18b	-	200	200	200	200	-	-	200	200
18c	100	200	-	50	200	200	200	200	200
18d	200	200	-	200	200	200	200	200	-
Ciprofloxacin	25	25	25	25	25	25	25	N.A.	N.A.
Ketoconazole	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	25	25

N.A. = not active

MS, m/z (%): 430 [M^+ + 4] (10), 60 [C_3H_8O] (100); Analysis: calcd. for $C_{25}H_{22}N_4O_3$ (426.47): C, 70.41; H, 5.20; N, 13.14%; found: C, 70.59; H, 5.40; N, 13.26%.

General procedure for the synthesis compounds 17a, 17b

A mixture of compound **5a** (0.40 g, 0.001 mol), and an appropriate acid anhydride, namely: succinic anhydride and/or phthalic anhydride (0.001 mol) in acetic acid (10 mL) was refluxed for 6 h. The formed precipitate was filtered, dried and recrystallized to give the title compound **17a**,**b**, respectively.

2-(2,5-Dioxo-2H-pyrrol-1(5H)-ylamino)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (17a)

Yield 63%; m.p. 149-150°C; IR (KBr, cm^{-1}): 3256 (NH), 2929, 2854 (CH_2 - tetrahydronaphthalene protons), 2216 (CN), 1719 (2CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.71, 2.76 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.76 (6H, s, 2-OCH_3), 6.79 (2H, s, CH-pyrrole protons), 7.08-7.85 (7H, m, Ar-H and pyridine proton), 8.34 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 476 [M^+ - 4] (2), 54 [C_4H_6] (100); Analysis: calcd. for $C_{28}H_{24}N_4O_4$ (480.51): C, 69.99; H, 5.03; N, 11.66%; found: C, 69.74; H, 5.24; N, 11.52%.

2-(1,3-Dioxoisoindolin-2-ylamino)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (17b)

Yield 73%; m.p. 189-190°C; IR (KBr, cm^{-1}): 3139 (NH), 2922, 2850 (CH_2 - tetrahydronaphthalene protons), 2214 (CN), 1722 (2CO); ^{1}H NMR (DMSO-d_6, δ, ppm): 1.74, 2.75 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.79 (6H, s, 2-OCH_3), 6.93-7.94 (11H, m, Ar-H and pyridine proton), 8.51 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 501 [$C_{31}H_{25}N_4O_3$] (2), 76 [C_6H_4] (100); Analysis: calcd. for $C_{32}H_{26}N_4O_4$ (530.57): C, 72.44; H, 4.94; N, 10.56%; found: C, 72.20; H, 4.74; N, 10.69%.

General procedure for the synthesis of compounds 18a-d

A mixture of compound **5a** (0.40 g, 0.001 mol) and an appropriate aldehyde, namely: 4-chlorobenzaldehyde, anisaldehyde, 3-methylfurfural and/or 3-indolaldehyde (0.001 mol) in absolute ethanol (15 mL) was refluxed for 6 h. The reaction mixture was cooled and poured onto ice/cold water. The formed precipitate was filtered, dried and recrystallized

from methanol to give the title compound **18a-d**, respectively.

2-(2-(4-Chlorobenzylidene)hydrazinyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (18a)

Yield 48%; m.p. 111-112°C; IR (KBr, cm^{-1}): 3396 (NH), 2919, 2852 (CH_2 - tetrahydronaphthalene protons), 2215 (CN); ^1H NMR (DMSO-d_6, δ, ppm): 1.71, 2.76 (8H, m, 4(CH_2) – tetrahydronaphthalene protons), 3.80 (6H, s, 2-OCH_3), 7.01-7.98 (12H, m, Ar-H, CH=N and pyridine proton), 8.60 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 415 [M^+ - C_6HCl] (2), 165, 167 [$C_8H_6ClN_2$] (100, 31); Analysis: calcd. for $C_{31}H_{27}ClN_4O_2$ (523.02): C, 71.19; H, 5.20; N, 10.71%; found: C, 71.38; H, 5.31; N, 10.57%.

2-(2-(4-Methoxybenzylidene)hydrazinyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (18b)

Yield 55%; m.p. 120-121°C; IR (KBr, cm^{-1}): 3423 (NH), 2929, 2838 (CH_2 - tetrahydronaphthalene protons), 2214 (CN); ^1H NMR (DMSO-d_6, δ, ppm): 1.68, 2.61 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.79 (6H, s, 2-OCH_3), 3.82 (3H, s, -OCH_3), 7.05-8.03 (12H, m, Ar-H, CH=N and pyridine proton), 8.52 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 506 [$C_{31}H_{30}N_4O_3$] (2), 77 [C_6H_5] (100); Analysis: calcd. for $C_{32}H_{30}N_4O_3$ (518.61): C, 74.11; H, 5.83; N, 10.80%; found: C, 74.24; H, 5.91; N, 10.62%.

2-(2-((5-Methylfuran-2-yl)methylene)hydrazinyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (18c)

Yield 60%; m.p. 109-110°C; IR (KBr, cm^{-1}): 3422 (NH), 2921, 2851 (CH_2 - tetrahydronaphthalene protons), 2214 (CN); ^1H NMR (DMSO-d_6, δ, ppm): 1.72, 2.67 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 2.22 (3H, s, -CH_3), 3.79 (6H, s, 2-OCH_3), 5.82-7.95 (10H, m, Ar-H, CH=N and pyridine proton), 8.41 (1H, s, NH, exchangeable by D_2O); MS, m/z (%): 494 [M^+ + 2] (2), 79 [C_6H_7] (100); Analysis: calcd. for $C_{30}H_{28}N_4O_3$ (492.57): C, 73.15; H, 5.73; N, 11.37%; found: C, 73.30; H, 5.55; N, 11.12%.

2-(2-((1H-indol-3-yl)methylene)hydrazinyl)-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(3,4-dimethoxyphenyl)pyridine-3-carbonitrile (18d)

Yield 68%; m.p. 131-132°C; IR (KBr, cm^{-1}): 3361, 3201 (2NH), 2923, 2854 (CH_2 - tetrahydronaphthalene protons), 2216 (CN); ^1H NMR (DMSO-d_6, δ, ppm): 1.74, 2.67 (8H, m, 4(CH_2) - tetrahydronaphthalene protons), 3.80 (6H, s, 2-OCH_3), 6.87-7.87 (13H, m, Ar-H, CH=N and pyridine proton), 8.32, 9.21 (2H, s, 2NH, exchangeable by D_2O); MS, m/z (%): 496 [M^+ - OCH_3] (2), 144 [$C_9H_8N_2$] (100); Analysis: calcd. for $C_{33}H_{29}N_5O_2$ (527.62): C, 75.12; H, 5.54; N, 13.27%: found: C75.27; H, 5.62; N, 13.10%.

Antimicrobial studies

The compounds were individually tested against a panel of Gram positive and Gram negative bacterial pathogens, yeast and fungi (*Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea, Klebseilla pneumoniae, Pseudomonas aeruginosa*, *Escherichia coli, Saccharomyces cerevisiae and Candida albicans*). Antimicrobial tests were carried out by the agar well diffusion method (9) using 100 µL of suspension containing 1 × 10^8 CFU/mL of pathological tested bacteria and 1 × 10^6 CFU/mL of yeast spread on nutrient agar (NA) and Sabouraud dextrose agar (SDA), respectively. After the media had cooled and solidified, wells (10 mm in diameter) were made in the solidified agar and loaded with 100 µL of tested compound solution prepared by dissolving 200 mg of the chemical compound in 1 mL of dimethyl sulfoxide (DMSO). The inculcated plates were then incubated for 24 h at 37°C for bacteria and 48 h at 28°C for fungi. Negative controls were prepared using DMSO employed for dissolving the tested compound. (ciprofloxacin (50 mg/mL) and ketoconazole (50 mg/mL)) were used as standard for antibacterial and antifungal activity, respectively. Ciprofloxacin and ketoconazole were used as standards because they have abroad spectra antibiotic widely used. After incubation time, antimicrobial activity was evaluated by measuring the zone of inhibition against the test organisms and compared with that of the standard. The observed zone of inhibition is presented in Table 1. Antimicrobial activities were expressed as inhibition diameter zones in millimeters (mm). The experiment was carried out in triplicate and the average zone of inhibition was calculated.

Minimal inhibitory concentration (MIC) measurement

The bacteriostatic activity of the active compounds (having inhibition zones (IZ) = 16 mm) was then evaluated using the twofold serial dilution technique (10). Twofold serial dilutions of the tested compounds solutions were prepared using the proper nutrient broth. The final concentration of the solutions were 200,100, 50, 25 µg /mL. Each 5 mL

received 0.1 mL of the appropriate inoculums and incubated at 37°C for 24 h for bacteria and 48 h at 28°C for fungi. The lowest concentration showing no growth was taken as the minimum inhibitory concentration (MIC) (Table 2).

RESULTS AND DISCUSSION

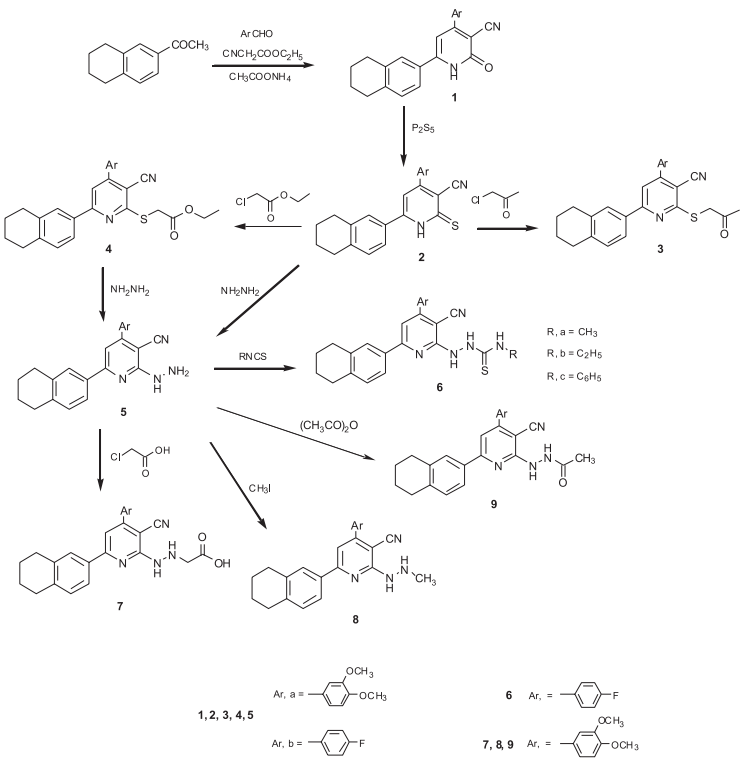
Chemistry

The target compounds were synthesized according to steps outlined in Schemes 1 and 2. The key intermediates 6-(1,2,3,4-tetrahydronaphthalen-6-yl)-2-mercapto-4-(3,4-substituted-phenyl) pyridine-3-carbonitrile **2a**,**b** were prepared by the treatment of 1,2-dihydro-6-(1,2,3,4-tetrahydronaphthalen-6-yl)-4-(substituted phenyl)-2-oxopyridine-3-carbonitrile **1a**,**b** with phosphorus pentasulfide in dry pyridine. Mass spectra of compounds **2a**,**b** showed molecular ion peak at 402 and 360, respectively.

Treatment of the key intermediates **2a**,**b** with chloroacetone and/or ethyl chloroacetate produced the corresponding derivatives **3a**,**b** and **4a**,**b**, respectively. IR spectra of compounds **3a**,**b** showed bands at 1702, 1709 cm^{-1} due to (CO) group and its ^1H NMR showed singlet signals at δ 2.28, 2.17 ppm due to methyl group and also singlet signals at δ 4.10, 4.12 ppm of methylene group. However, IR spectra of compounds **4a**,**b** showed bands at 1725 and 1740 cm^{-1} due to (CO) ester group and its ^1H NMR spectra exhibited the ester group signals at δ 1.29, 4.16 and 1.24, 4.13 ppm, respectively.

Condensation of **4a**,**b** with hydrazine hydrate afforded hydrazide derivatives **5a**,**b** and their IR spectra showed bands at 3245, 3177 and 3336, 3208 cm^{-1}, respectively, due to ($NHNH_2$) group. Compound **5a** was allowed to react with different substituted isothiocyanates, namely: methyl isothiocyanate, ethyl isothiocyanate and/or phenyl isothiocyanate, to give the corresponding substituted thiosemicarbazides **6a**-**c**. ^1H NMR of compound **6a** showed singlet signal at δ 2.56 ppm due to methyl group and **6b** showed signals at δ 1.05 and 3.51 ppm due to ethyl group.

Moreover, **5a** undergo reaction with chloroacetic acid to give the corresponding derivative **7** and its ^1H NMR showed singlet signal at δ 3.51 ppm due to methylene group. Also, reaction of compound **5a** with iodomethane gave the methylhydrazide derivative **8**. ^1H NMR showed singlet signal



Scheme 1. Synthesis of hydrazide pyridines of tetrahydronaphthalene

at δ 2.56 ppm due to methyl group. In addition, reaction of **5a** with acetic anhydride yielded acetohydrazide derivative **9**. ^{1}H NMR showed singlet signal at δ 2.21 ppm due to methyl group. (Scheme 1).

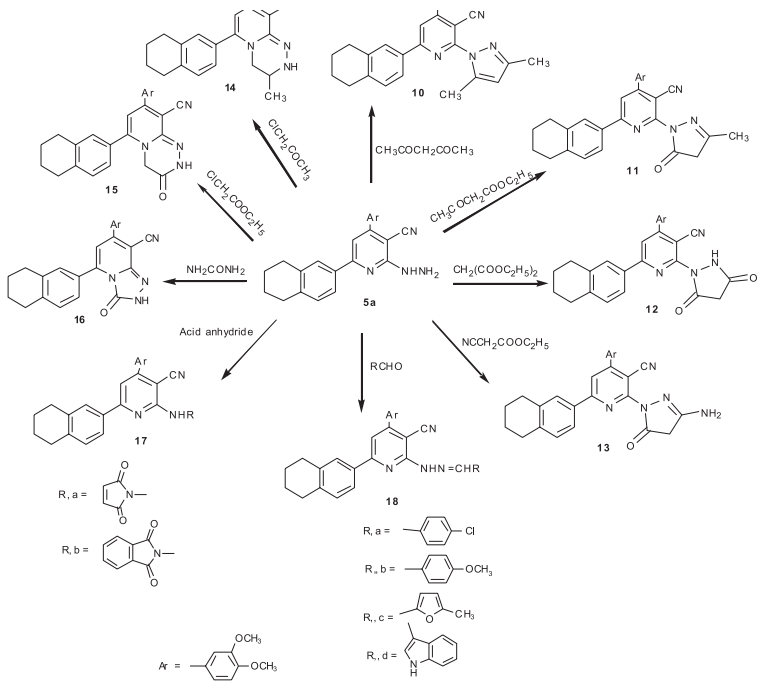
The hydrazide derivative **5a** is very useful intermediate for further cyclocondensation reactions. Thus, cyclocondensation of **5a** with active methylene derivatives, namely; acetylacetone, ethyl acetoacetate, diethylmalonate and ethyl cyanoacetate, yielded the corresponding pyrazole derivatives **10**-**13**, respectively. ^{1}H NMR spectrum of compound **10** showed singlet signals at δ 2.94 and 6.01 ppm due to methyl group and CH of pyrazole moiety. Also, ^{1}H NMR spectrum of compound **11** showed singlet signal at δ 2.02 and 2.21 ppm due to methyl group and CH_2 of pyrazolinone moiety. IR spectrum of compound **12** showed bands at 1735 and 1633 cm^{-1} due to the two carbonyl groups of pyrazoldione moiety. IR spectrum of compound **13** showed band at 1736 cm^{-1} due to CO group. In addition, cyclocondensation of **5a** with chloroacetone and/or ethyl chloroacetate gave the corresponding triazine derivatives **14** and **15**, respectively. ^{1}H NMR spectrum of compound **14** showed doublet signal at δ 1.13 ppm and multiple signals at δ 2.79, 3.34 ppm due to CH_3, CH_2 and CH groups of triazine moiety. IR spectrum of compound **15** showed band at 1740 cm^{-1} due to carbonyl group of triazine moiety. Also, cyclocondensation of **5a** with urea afforded the corresponding triazole derivative **16**.

Moreover, condensation of **5a** with acid anhydrides, namely: maleic anhydride and/or phthalic anhydride yielded pyrrole and isoindoline derivative **17a**,**b**. respectively. A series of new Schiff bases **18a**-**d** were synthesized by the condensation of hydrazide derivative **5a** with various substituted aromatic aldehydes, namely: 4-chlorobenzaldehyde, anisaldehyde, 5-methylfurfural and/or indole-3-aldehyde. The ^{1}H NMR spectrum of **18b** showed the three methoxy groups at δ 3.79 and 3.82 ppm while ^{1}H NMR of **18c** showed signal at δ 2.22 ppm of methyl group of furan ring.

Antimicrobial activity

Most of the newly synthesized compounds were evaluated for their *in vitro* antibacterial activity against *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* ATCC6633, *Bacillus megaterium* ATCC 9885 and *Sarcina lutea* as examples of Gram positive bacteria and *Klebsiella pneumoniae* ATCC 13883, *Pseudomonas. aeruginosa* ATCC 27953 and *Escherichia coli* ATCC 25922 as examples of Gram negative bacteria. They were also evaluated for their *in vitro* antifungal activity against *Saccharomyces cerevisiae* and *Candida albicans* NRRL Y-477.

Agar diffusion method was used for determination of the antimicrobial activity using ciproflox-



Scheme 2. Synthesis of different heterocycles of tetrahydronaphthalene

acin and ketoconazole as reference drugs. The results were recorded for each tested compounds as the average diameter as inhibition zones (IZ) of bacterial or fungal growth around the discs in mm (Table 1).

Minimum inhibitory concentration (MI) measurements were determined for the compounds (Table 2).

According to the obtained results (Tables 1, 2), it can be noticed that the parent starting compound 3,4-dimethoxyphenylpyridine **1a** and its thioxopyridine derivative **2a** produced weak to complete insensitivity towards the tested Gram positive and Gram negative bacterial strains. Also, the attachment of sulfur group of **2a** to ketone and ethyl ester side chains **3a**, **4a** did not improve the antibacterial activity. In comparison, the attachment of 4-fluorophenyl ring to the thioxopyridine ring in **2b** enhanced the activity of Gram positive and Gram negative bacterial growth inhibition to be half of that obtained by ciprofloxacin (MIC: 50, 25 μg/mL, respectively).

Broad spectrum of antibacterial activity greater than that obtained by the reference drugs was obtained upon the attachment of a methyl ketone side chain to the sulfur group of **2b** to get the derivative **3b** (MIC 18-22 μg/mL). A noteworthy enhancement in the activity against both Gram positive and Gram negative bacteria to be equal to that obtained by ciprofloxacin was gained by hydrazinolysis of the parent **2a** to give the hydrazine pyridine derivative **5a** (MIC: 25 μg/mL).

At the same time, the incorporation of a hydrazide side chain to the sulfur group **5b** produced sensitivity towards the tested Gram negative bacteria (MIC 25-50 μg/mL) but complete loss of activity against Gram positive bacterial strain, while the conversion of side chain to thiosemicarbazide **6a-c** enhanced the sensitivity of the compounds against Gram positive bacteria used in the experiment (MIC: 50-100 μg/mL), but unfortunately led to great reduction of the activity towards Gram negative bacteria.

It is noticeable that the incorporation of different heterocyclic rings to the parent 3,5-dimethoxypyridine ring such as oxadiazole, triazinephenyl, thiazolo, triazine, pyrazole and different substituted triazinephenyl rings did not offer any advantage in the antibacterial activity against both Gram positive and Gram negative bacteria.

The data obtained revealed that the listed compounds have weak antifungal activity except the parent 4-fluoropyridine derivative **2b**, which showed equipotent antifungal activity to that obtained by the reference ketoconazole (MIC: 25 μg/mL).

CONCLUSION

The antimicrobial evaluation of most of the newly prepared compounds exhibited that high broad spectrum activity against both Gram positive and Gram negative was gained by the parent 3,4-dimethoxypyridine derivative attached to hydrazine side chain **5a** and also by the parent thioxopyridine compound bearing 4-fluorophenyl group **2b** and its ketone derivative **3b**. Interestingly, **2b** is the only derivative that exhibited dual antibacterial and antifungal activity.

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SYNTHESIS AND STUDY OF HALOGENATED BENZYLAMIDES OF SOME ISOCYCLIC AND HETEROCYCLIC ACIDS AS POTENTIAL ANTICONVULSANTS

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Abstract: A series of potential anticonvulsants have been synthesized. There are eight fluorobenzylamides and three chlorobenzylamides of isocyclic or heterocyclic acids. Two not halogenated benzylamides were also synthesized to compare the effect of halogenation. The aim of the research performed was to evaluate whether halogenation of the mother structure is able to improve its anticonvulsant activity. The compounds were tested in Anticonvulsant Screening Project (ASP) of Antiepileptic Drug Development Program (ADDP) of NIH. Compound **1** showed MES ED_{50} = 80.32 mg/kg, PI = 3.16. Compound **7** showed CKM ED_{50} = 56.72 mg/kg. Compound **8** showed MES ED_{50} = 34.23 mg/kg and scPTZ ED_{50} > 300 mg/kg, PI = 8.53.Compound **13** showed 6Hz ED_{50} = 78.96, PI = 3.37. The results indicate that fluorination does not improve activity, whereas chlorination in our experiment even reduces it.

Keywords: anticonvulsants, chlorobenzylamides, fluorobenzylamides of isocyclic and heterocyclic acids

Many aromatic and heterocyclic acid benzylamides have been synthesized in search for new anticonvulsants (1-5). Some of them have been reported to be potent agents. Kohn et al. noticed significant role of benzyl fragment of these compounds (6, 7). They observed that electron-withdrawing substituents in benzyl moiety are able to increase anticonvulsant activity (8). The benzyl fragment could be also distinguished in the molecule of biphenyl, which derivatives are pharmacologically active affinity baits, chemical reactive units (9). The benzyl substituents change the hydrophobicity and molecular shape of the compounds, which determines their mechanism of action. Benzylamides of aromatic and heterocyclic acids are antagonists of excitatory amino acids. Up to now, no univocal data have been reported regarding the structure of the binding sites of the receptor. Therefore, a good way to obtain better anticonvulsants could be to modify the structure of the active compounds obtained earlier, which are mother compounds. Here, the purpose of the study was to evaluate if the incorporation of electron-withdrawing halogen substituent into the benzyl ring of previously obtained pharmacologically active compounds is able to improve the activity of the mother compounds. The design of new anticonvulsants can be assisted by comparison of the hydrophobicity and molecular shape of the synthesized halogenated compound with those properties of the mother compounds. Therefore, log P values of the partition coefficient between n-octanol and water of the compounds have been taken under consideration. As it was found previously, the optimal log P value of active benzylamide anticonvulsants ought to be > 0 and probably near to 3 (5). This scale is the best compromise between water and lipids partition of the designed anticonvulsants. Log P values of the halogenated compounds probably should not be excessively different from the values of the mother structures. Superimposing structures of halogenated compounds on the mother structures can, at least theoretically, let us evaluate if the receptor affinity of both is similar, thus providing additional information of structure elements crucial for phar-

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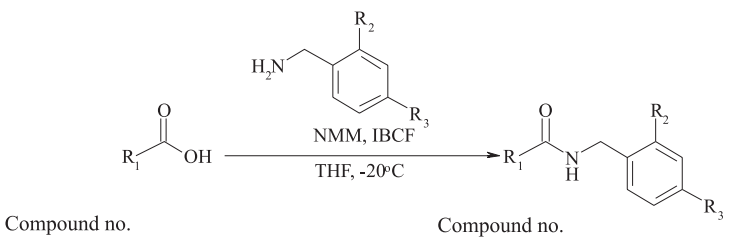
macological activity. The change of affinity could increase as well as decrease the pharmacological action of the new synthesized compounds in comparison to the mother structures.

EXPERIMENTAL

Chemistry

All used acids: 3-oxocyclopentanecarboxylic acid, 2-oxocyclopentaneacetic acid, coumarin-3-carboxylic acid, isoquinoline-3-carboxylic acid, 1-cyclopentenecarboxylic acid, picolinic acid N-oxide, 5-methylfurancarboxylic acid, isoquinoline-1-carboxylic acid and furan-2-carboxylic acid were purchased from Aldrich. Other reagents as isobutyl chloroformate, N-methylmorpholine, benzylamine, 2-chlorobenzylamine, 2-fluorobenzylamine and 4-fluorobenzylamine were supplied by Merck. DMF and THF were from POCH Gliwice.[1]H NMR spectra were recorded on a Bruker DM 400 MHz spectrometer. Chemical shifts were measured as δ units (ppm) relative to tetramethylsilane. TLC was carried out on a 0.25 mm thickness silica gel plates (Merck Kieselgel 60 F-254). The spots were visualized in UV light or with 0.3% ninhydrin in EtOH (97 : 3). The solvent system used in TLC was $CHCl_3$/MeOH in different ratios. HPLC was performed on a Shimadzu chromatograph equipped with LC-10AT pump, SPD-10A UV spectrophotometer and a computer registrator/recorder (CHROMA POLLAB, Warszawa). The peaks were recorded at 210 nm. Elemental analysis was performed on an Elementar Analysensysteme GmbH – vario El III Element Analyzer. Melting points were determined in a Böetius apparatus.



1. R_1 = R_2 = H R_3 = H
2. R_1 = R_2 = H R_3 = H
3. R_1 = R_2 = Cl R_3 = H
4. R_1 = R_2 = Cl R_3 = H
5. R_1 = R_2 = Cl R_3 = H
6. R_1 = R_2 = H R_3 = F
7. R_1 = R_2 = F R_3 = H
8. R_1 = R_2 = F R_3 = H
9. R_1 = R_2 = F R_3 = H
10. R_1 = R_2 = H R_3 = F
11. R_1 = R_2 = H R_3 = F
12. R_1 = R_2 = H R_3 = F
13. R_1 = R_2 = H R_3 = F

Scheme 1. Synthesis of compounds **1**-**13**

Synthesis of amides

Compounds **1-13** were synthesized using the mixed anhydrides method of peptide synthesis (10). Suitable acid (10 mmol) was dissolved in DMF (15 mL) and THF (15 mL) was added. Next, N-methylmorpholine (10 mmol, 1.1 mL) was added and the mixture was stirred under nitrogen and chilled to -15°C. Isobutyl chloroformate (10 mmol, 1.3 mL) was added dropwise to keep the temperature below -15°C. Then, benzylamine or halogenated benzylamine (10 mmol) in THF was added in small portions and the reaction mixture was stirred at -15°C for 30 min, at room temperature for 1 h. The solution was concentrated *in vacuo* and the residue was dissolved in EtOAc (20 mL). This solution was washed with 20 mL portions of 1 M HCl, saturated $NaHCO_3$ solution and saturated NaCl solution, then dried with anhydrous $MgSO_4$, filtered and concentrated *in vacuo*. The obtained compounds were purified by crystallization with EtOAc/hexane or MeOH/Et_2O. All stages of the synthesis were controlled by TLC. The purity of the final compound was determined by HPLC and identity by ^{1}H NMR. The pathway for the synthesis of the obtained compounds is shown in Scheme 1.

Computer calculations

The HyperChem 4.5 (Hypercube, Inc.) program was used. The semiempirical method PM 3 was applied for a single point calculation. Geometry optimization was performed by the Polak-Ribiere algorithm. Afterward, the QSAR Properties module using atomic parameters derived by Ghose et al. (11) was applied to calculate log P values as a measure of the hydrophobicity of the optimized structures of the compounds. RMS Fit module was used for overlapping the molecular structures of the compounds.

Pharmacology

All the synthesized compounds (**1-13)** were evaluated qualitatively in anticonvulsant identification system (ASP) – 6 Hz 32 mA and TOX tests. Compounds **1**, **2** and **5**-**13** were evaluated also in MES test (Table 3). The MES and TOX tests were performed in mice after *i.p*. administration, according to the method described by Krall et al. (12). The minimal clonic seizure (6 Hz 32 mA) test was used to assess compound's efficacy against electrically induced seizures but using a lower 6 Hz frequency and longer duration time – 3 s. The test was accomplished by the Barton et al. method (13). This test was performed, because some compounds ineffective in standard MES or scPTZ tests still have anticonvulsant activity *in vivo*. All the synthesized compounds (**1**-**13**) were evaluated qualitatively also in rats after *i.p*. and *p.o*. administration in MES and TOX tests (Table 4). The tests were performed according to the method of Krall et al. (12). Some

Table 1. Physical and analytical data of the synthesized compounds.

Compound No.	Formula	M.w.	M.p. °C	log. P*
1	$C_{13}H_{15}NO_2$	217.26	87-89	1.95
2	$C_{14}H_{17}NO_2$	231.30	101-102	2.35
3	$C_{17}H_{13}ClN_2O$	296.71	110-111	3.84
4	$C_{17}H_{12}ClNO_3$	313.75	186-188	3.19
5	$C_{17}H_{13}ClN_2O$	296.75	133-134	3.84
6	$C_{13}H_{14}FNO$	219.26	111-114	2.74
7	$C_{13}H_{14}FNO$	219.26	86-87	2.74
8	$C_{13}H_{11}FN_2O_2$	246.24	123-124	1.21
9	$C_{14}H_{16}FNO_2$	249.30	123-125	2.49
10	$C_{14}H_{16}FNO_2$	249.30	107-108	2.49
11	$C_{13}H_{12}FNO_2$	233.25	74-76	0.91
12	$C_{17}H_{13}FN_2O$	280.30	68-70	3.47
13	$C_{12}H_{10}FNO_2$	219.22	119-120	1.35

HPLC purity of the all compounds was 100%. The elemental analyses were within ± 0.4% of the theoretical value. * Hydrophobicity of the compounds is expressed as log P value calculated by a computational method.

Table 2. ^{1}H NMR spectra in $CDCl_3$ of the synthesized compounds.

Compound	Chemical shift in δ (ppm) in *CDCl$_3$*
1	2.11-2.27 (m, 3H), 2.31-2.64 (m, 4H), 2.84-2.92 (m, 1H CH), 4.46 (d, *J* = 5.0 Hz 2H, CH_2), 5.90 (s 1H, NH), 7.26-7.35 (m, 4H).
2	1.35-1.81 (m, 4H), 2.08-2.19 (m, 2H), 2.38-2.46 (m, 1H, CH), 4.45-4.60 (m, 2H), 7.22-7.37 (m, 8H).
3	4.83 (d, *J* = 5 Hz, 2H, CH_2), 7.22-7.26 (m, 2H, 2×CH), 7.38-7.41 (m, 1H, CH) 7.52-7.56 (m, 1H, CH), 7.69-7.79 (m, 2H, 2×CH), 7.87 (t, *J* = 4.1 Hz, 2H, 2×CH) 8.48 (d, *J* = 5.7 Hz, 1H, CH), 8.79 (s, br, 1H, NH), 9.57 (d, *J* = 8.6 Hz, 1H, CH).
4	4.75 (d, *J* = 7.5 Hz, 2H, CH_2), 7.22-7.26 (m, 2H), 7.35-7.46 (m, 5H), 7.64-7.70 (m, 3H), 8.39 (s, 1H, CH), 9.29 (s, br, 1H, NH).
5	4.83 (d, *J* = 4.7 Hz, 2H, CH_2), 7.20-7.26 (m, 2H, 2×CH), 7.40 (d, *J* = 9 Hz, 1H,CH), 7.50 (d, *J* = 9 Hz, 1H, CH), 7.71 (t, *J* = 6.0 Hz, 1H, CH), 7.75 (t, *J* = 6.0 Hz, 1H, CH),7.98-8.06 (m, 2H, 2xCH), 8.64 (s, 1H, CH), 8.69 (s br, 1H, NH), 9.16 (s, 1H, CH).
6	1.94-2.04 (m, 2H, CH_2), 2.45-2.55 (m, 4H), 4.47 (d, *J* = 4.8 Hz, 2H, CH_2), 5.88 (s br, 1H, NH), 6.56 (t, *J* = 5.0Hz, 1H, CH), 7.00 (t, *J* = 6.5 Hz 2H, CH_2), 7.25-7.29 (m, 2H).
7	1.94-2.03 (m, 2H, CH_2), 2.45-2.59 (m, 4H, 2×CH_2), 4.54 (d, *J* = 5.0, 2H, CH_2), 5.97 (s br, 1H, NH), 6.55 (t, *J* = 6.5, 1H, CH), 7.08 (q, 2H, 2xCH), 7.23-7.30 (m, 1H, CH), 7.37 (t, *J* = 6.0, 1H, CH).
8	4.74 (d, *J* = 5.4 Hz, 2H, CH_2), 7.0-7.13 (m, 2H, 2×CH), 7.22-7.29(m, 1H, CH), 7.36-7.48 (m, 3H, 3×CH), 8.24 (d, *J* = 6.0 Hz, 1H, CH), 8.46 (d, *J* = 5.5 Hz, 1H, CH), 11.6 (s br, 1H, NH).
9	1.33-1.44 (m, 1H), 1.48-1.60 (m, 1H), 1.67-1.83 (m, 3H), 2.11-2.19 (m, 2H), 2.40-2.49 (m, 1H), 2.80-2.89 (m, 1H), 3.45 (s br, 1H), 4.48-4.69 (m, 2H, CH_2), 6.99-7.12 (m, 2H), 7.20-7.27 (m, 1H), 7.39-7.44 (m, 1H).
10	1.33-1.50 (m, 2H), 1.61-1.75 (m, 3H), 2.05-2.18 (m, 2H), 2.43 (q, *J* = 2 Hz, 1H), 2.71-2.81 (m, 1H), 3.44 (s br, 1H, NH), 4.46 (q, 2H, CH_2), 6.98 (t, *J* = 1.Hz, 2H, CH_2), 7.26-7.35 (m, 2H).
11	2.32 (s, 3H, CH_3), 4.56 (d, *J* = 5.5 Hz, 2H, CH_2), 6.09, 6.10 (dd, *J* = 0,6 Hz, 0.6 Hz, 1H, CH), 6.59 (s br, 1H, NH), 6.98-7.06 (m, 4H), 7.26-7.34 (m, 2H).
12	4.71 (d, *J* = 7.7 Hz, 2H, CH_2), 7.03 (t, *J* = 6.0 Hz, 2H, 2×CH), 7.35-7.40 (m, 2H), 7.68-7.81 (m, 2H), 8.01 (t, *J* = 5 Hz, 2H, 2×CH), 8.57 (s br, 1H, NH), 8.65 (s, 1H, CH), 9.14 (s, 1H, CH).
13	4.58 (d, *J* = 6.0 Hz, 2H, CH_2), 6.50-6.52 (m, 1H, CH), 6.64 (s br, 1H, NH), 7.00-7.06 (m, 2H, 2×CH), 7.14 (d, *J* = 3.7 Hz, 1H, CH), 7.29-7.34 (m, 2H, 2×CH), 7.42 (d, *J* = 1.5 Hz, 1H, CH).

Table 3. Anticonvulsant identification. Qualitative tests in mice, *i.p.*

Compound No.	MES, 0.5 h		TOX, 0.5 h		6 Hz, 0.5 h	
	mg/kg	prot./used	mg/kg	prot./used	mg/kg	prot./used
1	100	3/4	300	6/8	300	4/4
2	300	4/4	300	3/8	100	2/4
3	ND	ND	100	0/4	100	1/4
4	ND	ND	100	0/4	100	0/4
5	300	0/4	300	1/8	300	0/4
6	300	4/4	300	0/8	300	4/4
7	100	4/4	300	7/8	100	2/4
8	300	4/4	300	1/8	300	3/4
9	300	0/4	300	0/8	300	2/4
10	300	4/4	300	8/8	300	4/4
11	100	2/4	300	6/8	100	3/4
12	100	1/4	300	0/8	300	3/4
13	300	4/4	300	0/8	100	2/4

MES = maximal electroshock seizure test. 6Hz = psychomotor minimal clonic seizure test, model 32 mA (ASP).TOX = neurological toxicity rotorod test. ND = not determined.

Table 4. Anticonvulsant identification. Qualitative tests in rats.

Compound No	*p.o.*, MES, 0.5 h		*p.o.*, TOX, 0.5 h.		*i.p.*, MES, 0.5 h		*i.p.*, TOX. 0.5 h	
	mg/kg	prot./used	mg/kg	prot./used	mg/kg	prot./used	mg/kg	prot./used
1	30	1/4	30	0/4	30	0/4	30	0/4
2	30	0/4	30	0/4	30	0/4	30	0/4
3	ND	ND	ND	ND	ND	ND	ND	ND
4	ND	ND	ND	ND	ND	ND	ND	ND
5	30	0/4	30	0/4	30	1/4	30	0/4
6	30	0/4	30	0/4	30	0/4	30	0/4
7	ND	ND	ND	ND	ND	ND	ND	ND
8	ND	ND	ND	ND	ND	ND	ND	ND
9	30	0/4	30	0/4	30	0/4	30	0/4
10	30	1/4	30	0/4	30	0/4	30	0/4
11	30	0/4	30	0/4	30	0/4	30	0/4
12	30	0/4	30	0/4	30	0/4	30	0/4
13	30	0/4	30	0/4	30	1/4	30	0/4

MES = maximal electroshock seizure test. TOX = behavioral positioned sense test. prot. = protected. ND = not determined.

Table 5. ED_{50} values.

Compound No.	Test	ED_{50} mg/kg	TD_{50} mg/kg	Time (h)	PI
1	MES*	80.32		0.25	3.16
	TOX		253.89	0.25	
7	CKM**	56.72		0.5	
8	MES***	34.23		0.25	8.53
	TOX		291.86	0.25	
	sc PTZ	300		0.25	
13	6 Hz****	78.96		0.5	3.37
	TOX		266.05	24.0	

MES = maximal electroshock test. TOX = neurological toxicity test. TD_{50} value determined from the rotorod test. PTZ = pentetrazole. PI = TOX TD_{50}/MES ED_{50} or TOX TD_{50}/6Hz ED_{50}. *Quantitative, mouse (*i.p.*) test (14). ** Quantitative corneal kindled mouse (CKM) test (15).*** Quantitative, mouse (*p.o.*) test (14). ****Quantitative, mouse 6Hz 32 mA (*i.p.*) test (13).

compounds were chosen for further examination. They were tested quantitatively in mice in MES and TOX tests according to the method of Swinyard et al. (14). ED_{50} values of these compounds were determined by the White method (15) and are given in Table 5. The biological responses of compounds **1**, **7**, **8** and **13** are given in Tables 6-9. Moreover, the evaluation of compounds **1** and **7** was accomplished in the pilocarpine induced status prevention test (ASP) using the method of Racin et al. (16). In this test, the compounds were assessed for evaluation of potential activity against nerve agents using the pilocarpine model of epilepsy as the introductory screen. The compound tested was administered to rat *i.p.*. Then, a challenge dose of pilocarpine was administered to observe the treatment effects of the compound. The outcome measures are determined in protected or non-protected animals. The obtained results are given in Table 10.

RESULTS

A series of halogenated benzylamides of heterocyclic and isocyclic acids was synthesized (**1**-**13**).

Physico-chemical data of the synthesized compounds are given in Tables 1 and 2, the results of preliminary pharmacological tests in Tables 3–10. Compound **1** showed MES ED_{50} = 80.32 mg/kg, PI = 3.16. Compound **7** showed in quantitative corneal kindled mouse test CKM ED_{50} = 56.72 mg/kg. Compound **8** showed MES ED_{50} = 34.23 mg/kg and scPTZ ED_{50} = 300 mg/kg, PI = 8.53. Compound **13** showed 6Hz ED_{50} = 78.96, PI = 3.37.

DISCUSSION

A series of isocyclic and heterocyclic acids benzylamides were previously obtained in search for new effective anticonvulsants (1-5). Preliminary anticonvulsant identification tests (ASP) allow to define the structure elements of this group of compounds conditioning pharmacologically advantageous properties. It was found that 5-membered

Table 6. Biological response of **1** (mice *i.p.*).

Test	Time (h)	Dosage (mg/kg)	Protected/used
MES	0.25	50	0/8
MES	0.25	75	4/8
MES	0.25	100	6/8
MES	0.25	150	8/8
scPTZ	0.25	255	2/8
TOX	0.25	150	0/8
TOX	0.25	225	2/8
TOX	0.25	260	3/8
TOX	0.25	300	8/8

MES = maximal electroshock test. TOX = neurological neurotoxicity test. PTZ = pentetrazole.

Table 7. Biological response of **7** (corneal kindled mouse).

Test	Time (h)	Dosage (mg/kg)	Protected/used
CKM	0.5	25	0/8
CKM	0.5	50	3/8
CKM	0.5	65	5/8
CKM	0.5	100	8/8

CKM = corneal kindled mouse.

Table 8. Biological response of **8** (mice *p.o.*).

Test	Time (h)	Dosage (mg/kg)	Protected/used
MES	0.25	10	0/8
MES	0.25	30	4/8
MES	0.25	50	5/8
MES	0.25	70	8/8
scPTZ	0.25	300	0/8
TOX	0.25	150	1/8
TOX	0.25	200	1/8
TOX	0.25	300	3/8
TOX	0.25	500	8/8

MES = maximal electroshock test. TOX = neurological neurotoxicity test. PTZ = pentetrazole.

Table 9. Biological response of **13** (6 Hz, mice, *i.p*).

Test	Time (h)	Dosage (mg/kg)	Protected/used
6Hz	0.50	35	1/8
6Hz	0.50	65	4/8
6Hz	0.50	85	4/8
6Hz	0.50	150	6/8
6Hz	0.50	250	4/4
TOX	24.0	150	1/8
TOX	24.0	210	1/8
TOX	24.0	300	3/8
TOX	24.0	500	8/8

MES = maximal electroshock test. TOX = neurological neurotoxicity test. PTZ = pentetrazole

Table 10. Pilocarpine-induced status test in rats. Response data.

Compound No.	Dosage (mg/kg)	Time	Protected/used
1	400	0.0	0/7
7	400	0.0	0/8

structure of acid is especially useful. Substituents of the ring, presence of heteroatoms or double bonds do not have a decisive influence upon the activity. However, among the benzylamides of 6-membered acids and bicyclic 6-membered acids there were also some compounds with poor anticonvulsant activity. Electron-withdrawing groups on benzylamide site are able to increase the anticonvulsant activity. Therefore, it was decided to halogenate some previously obtained most active structures. Also there were obtained six fluorinated benzylamides of isocyclic 5-membered acids (**6**, **7**, **9**, **10**, **11 and 13**) and one fluorinated benzylamide of 6-membered heterocyclic acid (**8**). Also there were obtained one fluorinated (**12**) and three chlorinated (**3**, **4**, **5**) benzylamides of bicyclic 6-membered acid. It was decided to synthesize two new benzylamides of isocyclic acid (**1**, **2**) and to fluorinate them (**9**, **10**) for comparison of the activity. All the synthesized compounds were screened in qualitative tests (ASP): in mice, *i.p*. (MES, 6 Hz 32 mA, TOX) (Table 3) and in rats, *p.o*. and *i.p*. (MES, TOX) (Table 4). On the basis of the preliminary results some compounds (**1**, **7**, **8** and **13**) were put to further tests. There were performed the following tests (ASP): ED_{50} values determination (Table 5), biological response determination (Tables 6-9), pilocarpine-induced status, and response data (Table 10). The results lead to the conclusion that the fluorination of the active mother structure does not improve anticonvulsant activity (similar activity of compounds **6**-**13** and mother compound (N-oxidepicolinic acid benzylamide (2), 1-cyclopentenecarboxylic acid benzylamide (4), 2-furoic acid benzylamide (4), 5-methyl-2-furoic acid benzylamide (4), 3-isoquinolinecarboxylic acid benzylamide (4), 2-oxocyclopentaneacetic acid benzylamide (**2**)). All the obtained fluorinated compounds show strong anticonvulsant activity and low neurotoxicity. On the other hand chlorination of the mother compounds yields derivatives of decreased activity or not active at all (high activity of mother compound - 3-isoquinolinecarboxylic acid benzylamide (4) and lack of activity of compounds **4** and **5**). The important property of the structure related to biomolecular interaction is hydrophobicity of the compound. Its pertinent value assures a better transmembrane transport, protein binding and receptor affinity of the compound. The values of log P of the synthesized halogenated compounds reported in Table 1 approach the values of the mother compounds: (1-cyclopentenecarboxylic acid benzylamide – 2.60 (4), 3-cyclopentenecarboxylic acid benzylamide – 2.54 (4), cyclopentanocarboxylic acid benzylamide – 2.80 (4), 2-furoic acid benzylamide – 1.21 (5), 5-methyl-2-furoic acid benzylamide – 0.77 (5), 3-isoquinolinecarboxylic acid benzylamide – 3.33 (5), 2-oxycyclopentaneacetic acid benzylamide – 2.35 (**2**). This confirms relations

among the hydrophobicity and anticonvulsant activity of the compounds. However, it does not explain the difference between the activity of fluorinated and chlorinated structures. The molecular shape is the major factor contributing to the receptor affinity. The test of structure overlapping was performed to study the space relations between the halogenated derivatives and the active mother compounds. The optimized structures of the synthesized halogenated compounds were superimposed on the mother structures. Generally, a good superimposing was observed. It suggests that the receptor affinity of the mother and the newly synthesized compounds is similar and therefore, pharmacological activity is also similar, which was confirmed by the results of pharmacological tests. However, there is no explanation at the moment of difference between activity of fluorinated and chlorinated structures. Among the synthesized and tested compounds the most promising are **1**, **7**, **8** and **13**. The PI values of these compounds is enclosed in limits 8.53 and 3.16. For comparison – PI values of known anticonvulsant drugs: valproic acid is 6.0 (MES ED_{50} = 425.1 mg/kg, TD_{50} = 243.0), phenobarbital is 6.7 (MES ED_{50} = 9.1 mg/kg, TD_{50} = 6.7 mg/kg). What deserves particular attention is very low neurotoxicity of these four compounds, similar to valproic acid, but much lower than phenobarbital (**1** – TD_{50} = 253.89 mg/kg, **7** – TD_{50} = 312.04 mg/kg, **8** – TD_{50} = 291.86 mg/kg and **13 -** TD_{50} = 266.05 mg/kg) (Table 5). High anticonvulsant activity and low neurotoxicity also show biological response of these compounds (Table 6-9). Considering the structure-activity relationship it is worth observing that three promising compounds are derivative of 5-membered acids (**1**, **7**, **13**) and three (**7**, **8**, **13**) are fluorinated benzylamides. This observation is worth consideration in the course of new anticonvulsant designing.

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NATURAL DRUGS

HEMATOLOGICAL AND IMMUNOBIOCHEMICAL STUDY OF GREEN TEA AND GINGER EXTRACTS IN EXPERIMENTALLY INDUCED DIABETIC RABBITS

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Abstract: The present study was designed to investigate the effects of the extract of green tea and/or ginger on some hematological and immunobiochemical profiles in alloxan-induced diabetic rabbits. The results revealed that treatment of diabetic animals with extract of green tea and/or ginger elevated the decreased HDL-c and LDL-c but significantly decreased triglycerides, the elevated glucose and GOT concentrations. The result also displayed a non-significant increase in the levels of CRP and fibrinogen. The experiment also revealed that the elevated MDA and GSH level fell down to the normal control group. The result also showed that after green tea and/or ginger extract treatment, the lowered RBC, WBC counts, PCV, percentage of neutrophils were increased and the elevated MCV, MCH, and MCHC of diabetic rabbits were decreased to normal levels. Thus, the overall results may indicate that green tea and/or ginger extracts have a significant hypoglycemic effect in diabetic rabbits. In addition, the extracts may be capable of improving hyperlipidemia, the impaired kidney function and hemogram in alloxan-induced diabetic rabbits.

Keywords: alloxan, ginger, green tea, hematology, immunobiochemical

Abbreviations: Hb - hemoglobin, PCV - packed cell volume, RBC - red blood cell, WBC - white blood cell, MCV - mean corpuscular volume, MCH - mean corpuscular hemoglobin, MCHC - mean corpuscular hemoglobin concentration

The World Health Organisation (WHO) reported that 300 million people would suffer diabetes mellitus (DM) by the year 2025 (1). The disease is considered as important public health problem, especially in developing and 3rd world countries due to inadequate treatment (2).

Although herbal medicines have long been used effectively in treating many diseases throughout the world, the mechanisms of most of the herbals used have not been defined. Many traditional plant treatments for diabetes are also used, but most of the evidence for their beneficial effects is anecdotal (3). Ginger (*Zingiber officinale*) has been used as a spice for over 2000 years. Its root and the obtained extracts contain polyphenol compounds: 6-gingerol and its derivatives, which have high antioxidant activity (4). Ginger's high antioxidant value has proved highly effective with its ability to scavenge a number of free radicals and protect cell membrane lipids from oxidation in a dose dependent manner (5). As in traditional medicine, rhizomes of ginger plants are consumed by women during ailment, illness and confinement. Ginger oil has been found to be an inhibitor of cyclooxygenase and lipoxygenase activities (6).

The medicinal properties of ginger were attributed to many active compounds in ginger. The major constituents in *Zingiber officinale* are the pungent vanilloids, gingerol, paradol, shogaols and zingerone (7). The antioxidant, antitumor, and anti-inflammatory pharmacologic effects of ginger were mainly attributed to these constituents (7).

Ginger acts as a hypolipidemic agent in cholesterol-fed rabbits (8). Also, it was reported (9) that

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ginger treatment significantly decreased both serum cholesterol and triglycerides. In addition, ginger decreased low density lipoprotein-cholesterol (LDL-cholesterol), very low density lipoprotein-cholesterol (VLDL-cholesterol) and triglycerides levels in apolipoprotein-E deficient mice (10). Furthermore, ethanolic extract of ginger prevented hypercholesterolemia and development of atherosclerosis in cholesterol-fed rabbits (11). It was found (12) that the ethanolic extract of ginger significantly reduced serum total cholesterol and triglycerides and increased the high density lipoprotein-cholesterol (HDL-cholesterol) levels; also, the extract can protect tissues from lipid peroxidation and exhibit a significant lipid lowering activity in diabetic rats. The study (13) revealed that serum and liver cholesterol decreased when ginger was administered to hypercholesterolemic rats.

Green tea is one of four types of tea (white, green, black, and oolong) that come from the plant *Camellia sinensis*. White tea is the least processed form of tea, while black tea leaves are fermented. Green tea leaves are steamed, not fermented and hence preserve more polyphenols (14). The beneficial effects of green tea are attributed to the polyphenols, particularly the catechins, which make up to 30% of the dry weight of green tea leaves. These catechins are present in higher quantities in green tea than in black or oolong tea, because of differences in the processing of tea leaves after harvest (15).

Green tea is a widely-consumed beverage and, for centuries, has been regarded to possess significant health-promoting effects (16). The health-promoting effects of green tea are mainly attributed to its polyphenol content. Green tea is a rich source of polyphenols, especially flavanols and flavonols, which represent approximately 30% dry weight of the fresh leaves (16). Catechins are the predominant flavanols and are mainly comprised of epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC) (17).

Hematological and biochemical analyses after treatment with plant extracts in experimental animals are among the important methods of assessing the safety and toxicity of these plant extracts in animals and human being as well. Earlier studies have been done for investigating the effect of plant extracts on hematology and biochemistry of experimental animals but none has been done for investigating the effect of combined extracts of both green tea and ginger in rabbits. Therefore; we measured some hematological parameters for evaluation of the effect of the extract of green tea and/or ginger in diabetic-induced rabbits.

EXPERIMENTAL

Animals

One month before the study, the animals were treated against endo- and ecto-parasites and coccidiosis. Fifty (50) adult male rabbits of the New Zealand white strain (weighing between 1100 - 1800 g) were selected and kept for about 14 days for acclimatization. All animals were housed in stainless cages under standard laboratory conditions (temperature: 22.5 + 3.58^{o}C; relative humidity: 50 ± 20%; air ventilation: 10–15 times per hour; artificial lighting: 12 h per day), and were allowed free access to normal balanced (standard) diet purchased from Cairo company (Commercial pelleted food contained a minimum of 88% dry matter, minimum 15% crude protein, maximum 17% cellulose, maximum 10% crude ash and minimum 2300 kcal/kg metabolic energy) and to water *via* an automatic water supply system. The animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH).

Preparation of plant extracts

Ginger extraction

Fresh rhizomes of ginger roots purchased from ordinary market were washed, peeled and 10 g weighed out and mashed with mortar and pestle. This was then extracted with 0.1 liter of distilled water and boiled for 5 min at 100^{o}C. The extract was filtered and administered 1 mL/kg orally (30).

Green tea extraction

Fifteen grams of tea leaves were soaked in 500 mL of boiling water for 30 min and were then filtered. Extracts were prepared fresh daily (31). The tested plants were used in aqueous extracts at concentration of 0.2 g/mL by soaking these plants in boiling water. After 5 min, each extract was filtered and the filtrate was orally administered to rabbits at a temperature of 37 ± 1^{o}C. This filtrate was provided to rabbits as their sole source of drinking water.

Experimental protocol

Fifty (50) adult male rabbits used in this study were divided into five experimental groups, each group consisted of 10 animals: Group 1 (G1): (control group), normal animals without treatment (standard); Group 2 (G2): diabetic group (diabetes induced by alloxan) without any treatment; Group 3 (G3): diabetic animals treated with green tea extract;

Group 4 (G4): diabetic animals treated with ginger extract; Group 5 (G5): diabetic animals treated with combined extract of both green tea and ginger.

Aqueous extracts of green tea and/or ginger were administered orally to rabbits daily using a gastric tube at dose rate of 1 mL/100 g body weight per day for 3 weeks (32). The control groups (normal and diabetic) were orally administered only with the same volume of isotonic NaCl.

Female rabbits were not used in our experiments to avoid the effect of female reproductive hormones on the measured parameters.

Induction of diabetes

The most common method to induce diabetes is using phloridzin (alloxan) or streptozotocin. Considering the insensitivity of rabbits to streptozotocin, alloxan was used to induce diabetes in this study. Alloxan causes extensive ß cell destruction in the pancreas during 18 to 24 h after injection, which leads to hyperglycemia. The reason for alloxan's selective toxicity is its structural resemblance to glucose and its mechanism of effect is producing free radicals since it can be deactivated by anti-oxidants. At the start of the experiment; the animals in the latter four groups were injected intravenously in the ear vein with 150 mg/kg of 10% alloxan (alloxan monohydrate, Sigma Chemicals Co., Egypt) dissolved in 10% solution in normal saline to induce diabetes. The control group was injected only with the same volume of isotonic NaCl as the diabetic groups received. Normal range of blood glucose in New Zealand white rabbit is ranged between 4.2-10.4 mmol/L (75-189 mg/dL with an average of 115 mg/dL) (33). Animals with blood glucose levels ≥ 200 mg/dL were included in the study (34). Alloxan induces diabetes through destructing Langerhans islands of the pancreas. Therefore, a large amount of insulin is released from the pancreas cells after the injection. In order to prevent hypoglycemic shock, during the first 24 h after the alloxan injection, the rabbits received 10% dextrose instead of water (35, 36). Blood glucose levels were assayed 3 days after alloxan treatment to determine which animals had become diabetic and the degree of hyperglycemia.

Collection of blood samples

At the end of the experimental period (3 weeks), blood samples were collected from experimental animals through the ear vein into a set of labeled sterile bottles containing K3-EDTA for hematological parameters determination with careful considerations to avoid hemolysis during sampling and transfer. Another set of tubes without K3-EDTA was used to collect blood, immediately covered, left to clot and centrifuged at 4000 × g for 15 min at room temperature for separation of serum that will be used for immunobiochemical parameters assays.

Immunobiochemical parameters

The levels of serum triglycerides, HDL-cholesterol and LDL-cholesterol were estimated according to methods described in (37). Lipid peroxidation (malondialdehyde concentration, MDA) and reduced blood glutathione (GSH) concentration were determined according to the methods of (38). Glucose level was measured by the method described by (39). Aspartate aminotransferase (AST; SGOT) and alanine aminotransferase (ALT; SGPT) were measured using commercial kits.

Immunoglobulins were measured using the method of (40). C-reactive protein (CRP) was measured using the method of (41). All other chemicals used were of analytical reagent grade.

Hematological parameters

The packed cell volume (PCV) was measured by the microhematocrit centrifuge (40). Hemoglobin (Hb) concentration was determined by the cyanomethemoglobin technique (42). The red blood cells (RBCs) and white blood cells (WBCs) counting methods were based on the dilution of obtained blood with diluting fluids (Hayem & Turke) in RBCs and WBCs counting pipettes (42). Individual cells were then counted in the counting chamber (hemocytometer). Giemsa's staining method was used for the differential count of WBCs.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance followed by Duncant's test (PC-stat computer program). Finally, least significant difference (L.S.D) was used to test the difference among treatments. Results were considered statistically significant when p was ≤ 0.05.

RESULTS

As shown in Figures 1, 2, alloxan produced significant hyperlipidemic action, where a significant ($p \leq 0.05$) increase was recorded in the levels of plasma triglycerides and LDL-c when compared with normal group, but HDL-c level was significantly ($p \leq 0.05$) decreased. However, post-treatment with extract of green tea and/or ginger ameliorated the hyperlipidemic action of alloxan on lipid profile by changing either up or down the

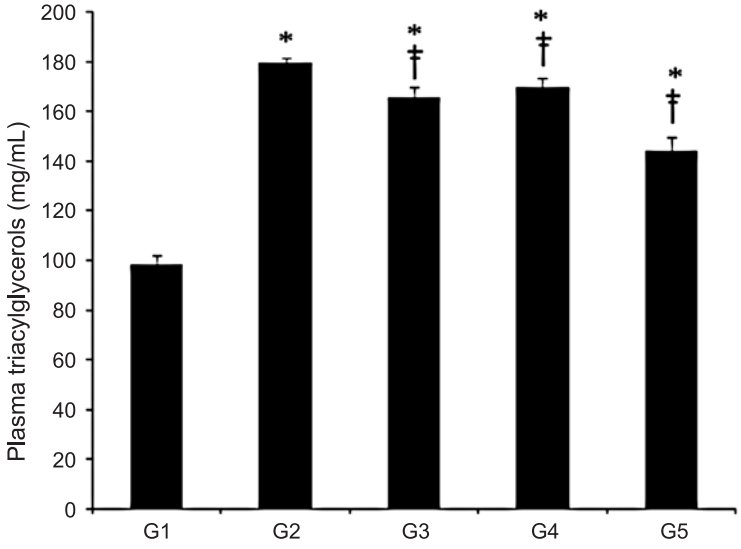

Figure 1. Plasma triacylglycerols in normal control, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

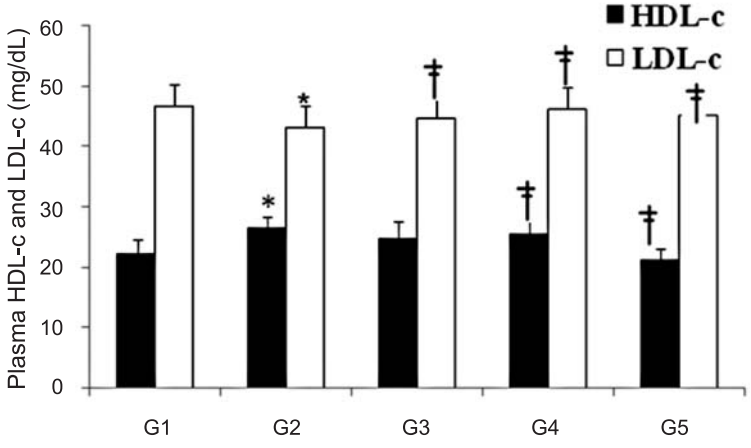

Figure 2. Plasma HDL-c and LDL-c levels in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

level to be normal or near the normal in case of HDL-c and LDL-c but decreasing significantly ($p \leq 0.05$) the level of triglycerides. In case of post-treatment by oral administration of both green tea and ginger extracts, the level of triglycerides decreased significantly ($p \leq 0.05$) more than the effect of post-treatment with oral administration of either green tea or ginger extract alone as shown in Figure 1.

Serum glucose concentration increased significantly ($p \leq 0.05$) in diabetic rabbits but treatment of diabetic rabbits with extract of green tea and/or ginger decreased the elevated glucose concentration significantly ($p \leq 0.05$); however, glucose concentrations were still significantly higher ($p \leq 0.05$) than those of the control group as shown in Figure 3.

Highly significant ($p \leq 0.05$) increase in both GPT and GOT was recorded in diabetic group compared to normal control group. Post-treatment of diabetic animals with extract of green tea or ginger returned their level to its normal level again. However, post-treatment of diabetic animals with extract of both green tea and ginger increased GOT significantly but not GPT as shown in Figure 4.

Regarding to MDA and GSH, alloxan-induced diabetic animals showed significant increase in MDA and a significant ($p \leq 0.05$) decrease in GSH levels compared to their equivalent levels in normal control animals as shown in Figure 5. However, post-treatment of diabetic animals with extract of either green tea or ginger resulted in significant ($p \leq 0.05$) increase in MDA as an indicator of lipid peroxidation process, while post-treatment of diabetic animals with extract of both green tea and ginger reduced significantly ($p \leq 0.05$) the level of MDA returning it to its normal control level. In case of GSH, post-treatment of alloxan-induced diabetic animals with extract of green tea and/or ginger return GSH level to its normal control animals.

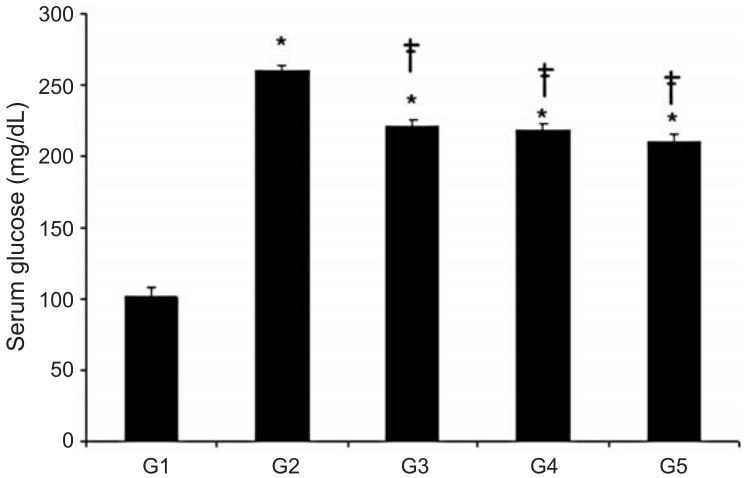

Figure 3. Serum glucose levels in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

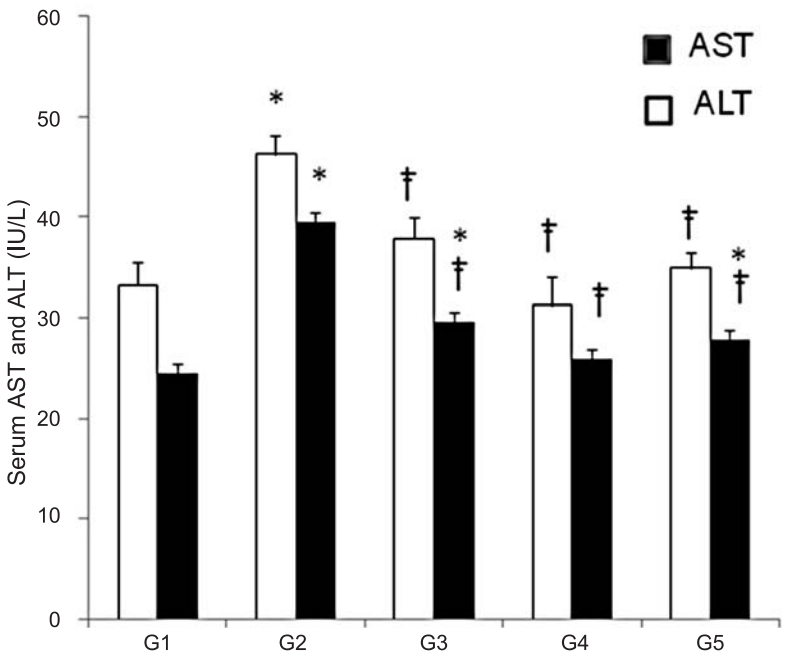

Figure 4. Serum AST and ALT levels in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

Regarding CRP and fibrinogen, alloxan-induced diabetic animals showed significant ($p \leq 0.05$) increase in the levels of CRP and fibrinogen, however, post-treatment of diabetic animals with extract of green tea and/or ginger showed slight but not significant ($p \leq 0.05$) increase in the levels of CRP and fibrinogen as shown in Figure 6.

Regarding immunoglobulins, alloxan decreased significantly IgG, IgM, and IgA compared to their levels in normal control group, as shown in Figure 7. Post-treatment of diabetic animals with either extract of green tea or ginger alone decreased significantly ($p \leq 0.05$) the levels of immunoglobulins. However, post-treatment of diabetic animals with a mixture extract of both green tea and ginger decreased significantly ($p \leq 0.05$) the immunoglobulins but in a lesser degree than groups post-treated with extract of either green tea or ginger alone, as shown in Figure 7.

As shown in Table 1, the results revealed that the RBC and WBC counts, PCV and neutrophil percentage decreased significantly ($p \leq 0.05$), while

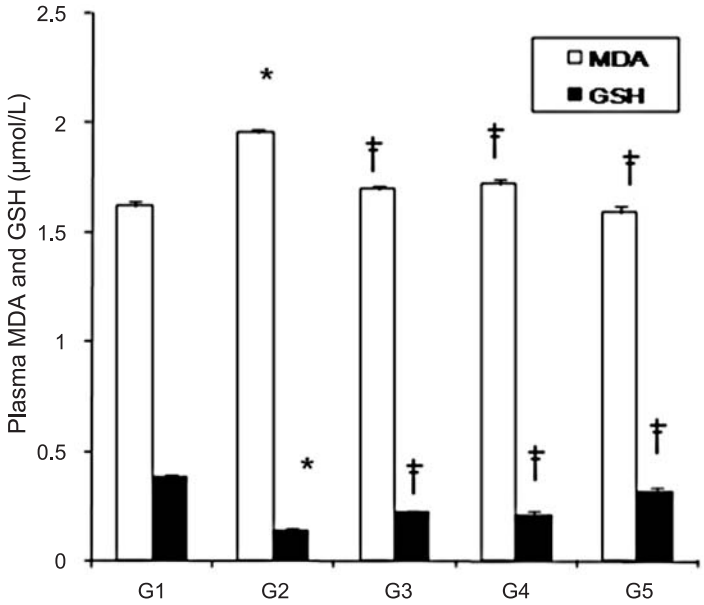

Figure 5. Plasma MDA and GSH levels in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

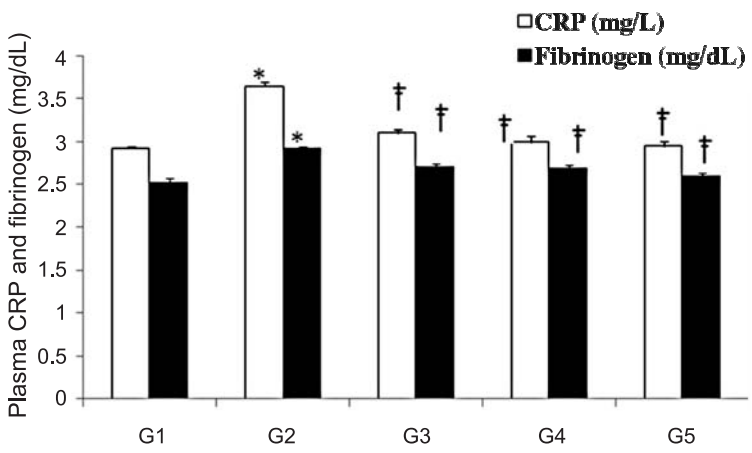

Figure 6. Plasma CRP and fibrinogen levels in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

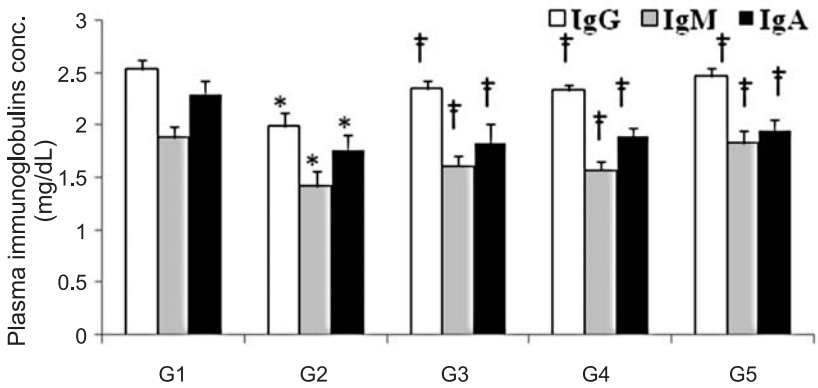

Figure 7. Plasma immunoglobulins concentrations in normal, diabetic and diabetic treated animals. Values are the mean ± SEM. * $p < 0.05$ *vs.* control group (G1) and ‡ $p < 0.5$ *vs.* diabetic group (G2)

Table 1. Comparative hematological values of normal control (G1), diabetic (G2) and diabetic treated animals (G3, G4, G5). Values are expressed as the mean ± SD. Means in the same row with different superscripts significantly differ ($p < 0.05$). Number of rabbits in each group = 10.

Parameter	**G1**	**G2**	**G3**	**G4**	**G5**
PCV (%)	37.00 ± 2.9^{a}	29 ± 2.5^{b}	33 ± 2.8^{c}	32 ± 2.9^{c}	36 ± 3.2^{a}
Hb (g/dL)	12.45 ± 0.9^{a}	12 ± 1.1^{b}	12.2 ± 1.2^{b}	12.4 ± 1.1^{a}	12.47 ± 0.9^{a}
RBCs ($\times 10^{6}$/mm^{3})	5.72 ± 0.4^{a}	4.1 ± 0.3^{b}	5.2 ± 0.1^{c}	5.4 ± 0.2^{a}	5.6 ± 0.2^{a}
MCV (μ^{3})	64.46 ± 5.4^{a}	70.73 ± 4.9^{b}	63.46 ± 5.1^{a}	59.26 ± 5.8^{c}	64.29 ± 5.2^{a}
MCH (pg)	21.6 ± 0.7^{a}	29.27 ± 1.2^{b}	23.46 ± 1.5^{a}	23 ± 1.2^{a}	22.26 ± 0.9^{a}
MCHC (%)	33.65 ± 1.8^{a}	41.37 ± 1.5^{b}	37 ± 1.8^{c}	38.75 ± 1.3^{c}	34.64 ± 0.8^{a}
WBCs ($\times 10^{3}$/mm^{3})	5.4 ± 0.5^{a}	3.1 ± 0.9^{b}	4.4 ± 0.6^{c}	4.8 ± 0.8^{d}	5.3 ± 0.6^{a}
Neutrophils (%)	30 ± 1.3^{a}	25 ± 1.5^{b}	29 ± 1.3^{a}	28 ± 1.2^{a}	33 ± 1.3^{c}
Lymphocytes (%)	61 ± 2.3^{a}	63 ± 2.1^{a}	61 ± 1.8^{a}	62 ± 1.7^{a}	60 ± 1.4^{a}
Monocytes (%)	4 ± 0.3^{a}	5 ± 0.4^{a}	4 ± 0.6^{a}	3 ± 0.5^{a}	4 ± 0.3^{a}
Eosinophils (%)	3 ± 0.2	4 ± 0.1	3 ± 0.1	4 ± 0.1	2 ± 0.2
Basophils	2 ± 0.1	3 ± 0.1	3 ± 0.1	3 ± 0.1	1 ± 0.1

PCV = packed cell volume; Hb = hemoglobin; RBC = red blood cell count; WBC = white blood cell count; MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration.

MCV, MCH, and MCHC increased significantly ($p \leq 0.05$) in alloxan-induced diabetic rabbits compared to normal control animals. Green tea and/or ginger extracts treatment increased ($p \leq 0.05$) the lowered RBC and WBC counts, PCV and neutrophil percentage in diabetic rabbits. However, green tea and/or ginger extracts treatment decreased the elevated MCV, MCH, and MCHC of diabetic rabbits to normal level. The other parameters as percentages of lymphocytes, monocytes, eosinophils and basophils showed non significant changes among normal and treated groups.

DISCUSSION

In the present study, statistical analysis revealed that alloxan-induced diabetic rabbits showed significant reduction as shown in the levels of serum triglycerides and LDL-c, but serum HDL-c level statistically increased (Figs. 1 and 2). However, post-treatment of diabetic animals with green tea or ginger extract ameliorated the lipid lowering action of alloxan, but in case of post-treatment with combination of green tea and ginger extracts, the level of triglycerides decreased significantly ($p \leq$

0.05) more than the effect of post-treatment with green tea or ginger extract alone. These findings are in agreement with earlier studies including (9, 12) and others. It was concluded that the hypocholesterolemic effect of ginger or green tea could have possibly resulted from the inhibition of cellular cholesterol biosynthesis after the consumption of the extract (10). Furthermore, it was reported (18) that the reduction of cellular cholesterol biosynthesis is associated with increased activity of the LDL receptor, which in turn leads to enhanced removal of LDL from blood, resulting in reduced serum or plasma cholesterol concentration. In our study, we can attribute this reduction in most lipid parameters to the severe lipolysis caused by both extracts to overcome the effect of insulin deficiency.

Referring to Figure 3, the alloxan induced diabetic treated groups (G3, G4 and G5) showed significant decrease ($p \leq 0.05$) in the level of glucose in comparison with diabetic untreated group (G2). In the present investigation, ginger and green tea extracts caused significant hypoglycemic effect in diabetic rabbits. Such a phenomenon of hypoglycemic response with green tea extract has already been reported (19). Figure 3 showed a significant increase in blood glucose levels in diabetic rabbits because of the destruction of pancreatic β cells by alloxan. The hypoglycemic effect of ginger increased gradually and was observed to be maximal at the end in group G5 when we used the two extracts together. The decrease in blood glucose levels was due to the antidiabetic compounds of ginger and different types of catechins present in green tea.

Regarding the effect of green tea and/or ginger extracts in alloxan-induced diabetic rabbits on the oxidant status and liver function enzymes; the present investigation showed that there were significant decreases ($p \leq 0.05$) in lipid peroxidation and elevated plasma total antioxidant capacity and blood reduced glutathione concentration in the treated groups 3, 4 and 5 in comparison with the diabetic group 2 (Figs. 4 and 5). This finding strongly confirms the antioxidant properties of ginger reported in previous investigations. Ginger has been reported to have a lowering effect on lipid peroxidation by influencing the enzymatic blood level of superoxide dismutase, catalase, and glutathione peroxidase (20). It has been also shown that ginger reduces cellular oxidation, scavenge superoxide anion and hydroxyl radicals. Ginger free phenolic and ginger hydrolyzed phenolic fractions exhibit free radical scavenging activity. Depletion of tissue GSH levels enhances cellular damage caused by oxidative stress (21). Significant depletion of GSH ($p < 0.05$) in diabetic rats suggests its increased utilization against reactive oxygen species (22). However, green tea and ginger treatment in diabetic rabbits reversed the GSH to normal levels, what shows that ginger has an antioxidant property. The antioxidative activity of green tea and ginger was attributed to scavenging superoxide anion and hydroxyl radicals by some phenols of ginger and catechins in green tea. Theoretically, our results may be due to the presence of many antioxidant compounds, phenolic ketone derivatives, catechins and volatile oils present in ginger and green tea.

Regarding the liver function enzymes, as shown in Figure 4, there were significant decreases ($p \leq 0.05$) in the activity of mean values of GOT and GPT activity in treated groups 3, 4 and 5 in comparison to group 2. These results go with those showed earlier (23) and attributed this disturbance in enzyme activity to lipid peroxidation produced as a result of diabetes. In the present work, the normalization of enzyme activity may be due to the effect that comes from the usage of the green tea and ginger extracts which leads to a lowering effect of free radicals on the cell membrane of liver cells. These results go with those given previously from GSH and MDA (38).

Statistically, as showed in Figure 6, fibrinogen and CRP both showed significant decrease ($p \leq 0.05$) in the treated groups 3, 4 and 5 when compared with diabetic group 2. The mean values of both fibrinogen and CRP showed significant increase ($p \leq 0.05$) in comparison with the control one. These findings come in line with that stated in (24) and (25). The obtained data clarify platelet inhibition (PI) found in the diabetic patient by revealing a significant interaction in elevated plasma fibrinogen in the presence of DM. In this investigation, the effect of green tea and ginger extract administration lead to decrease of inflammatory response caused by DM on endothelial cells. This was very clear in Figure 6, especially in group 5, due to the usage of both green tea and ginger extracts. This may be attributed to antiplatelet effect of different phenols and catechins present in both green tea and ginger.

Immunological status of the animals under experiment is shown in Figure 7. The statistical analysis of the obtained data revealed that there was a significant decrease ($p \leq 0.05$) in the mean values of immunoglobulins in diabetic group 2 in comparison with the control group 1. Administration of green tea and ginger extracts led to significant increase ($p \leq 0.05$) in treated groups 3, 4 and 5. The obtained results come in accordance with that stated

in (26). Theoretically, the obtained data can be attributed to fact that the ginger phenols and tea catechins may possess immunostimulant effects and this point needs further investigation, which may be our future research point.

Referring to Table 1 in the present study, which indicated that treatment with extracts of green tea and/or ginger might ameliorate some disturbed hematological parameters of diabetic rabbits, it has been suggested that anemia occurrence in DM is due to the increased non-enzymatic glycosylation of RBC membrane proteins, which correlates with hyperglycemia (27). Oxidation of these glycosylated membrane proteins and hyperglycemia in DM caused an increase in the production of lipid peroxides causing hemolysis of RBC.

Hematological indices are indicators and reflection of the effects of dietary treatments on animals in terms of the quality of feed ingested and nutrients available to the animal to meet its physiological requirements. The values of hemoglobin (Hb), an iron-containing conjugated protein that performs the physiological function of transporting oxygen and carbon dioxide, which didn't show any significant changes in diabetic or diabetic treated groups compared with those on the control diet group, suggest that the animals didn't suffered depressed respiratory capability at any group indicating that the oxygen-carrying capacity of the blood of the animals are not affected either in diabetic or diabetic treated groups. Thus, increased RBC count of green tea and/or ginger treated diabetic rabbits could be due to the lowered lipid peroxide level in RBC membrane leading to a decreased susceptibility of RBC to hemolysis. Since non-enzymatic glycosylations of membrane proteins correlate with hyperglycemia (27), it might be said that green tea and ginger extract produced their effect by decreasing the elevated glucose concentration in green tea and ginger extract treated diabetic rabbits. However, more studies by measuring the RBC fragility, and serum folic acid, iron, cobalt, vitamin B_{12} and calcium levels are needed to demonstrate the exact mechanism of action of green tea and ginger extracts on increased RBC count of diabetic rabbits. Therefore, our results suggest that the extracts of green tea and ginger stimulate the synthesis (erythropoiesis) and concentration of erythrocytes till normalizing RBC in anemic diabetic rabbits. The corresponding statistical decrease in the PCV (measure of the volume of blood consisting of solid cells) of the diabetic animals and its normalizing with green tea or ginger extract treatment suggest their role in erythropoiesis. Taken together, the results of RBC, Hb and PCV suggest that the extracts of green tea and ginger possess antioxidant properties and help in RBC membrane stabilization by binding to proteins and carbohydrates which are components of RBC membrane and therefore may prevent breakdown of RBC membrane and antagonize the anemic effect of alloxan.

Neutrophils ingest and kill bacteria and have been called the body's first line of defense against bacterial infections (28). It has been suggested that the body's defense mechanism against infections was disturbed due to the disturbed neutrophil function in diabetes (29). In this experiment, we demonstrated that treatment of diabetic rabbits with green tea and ginger extract increased the lowered neutrophil percentage of WBC to the control level. This result indicated that green tea and/or ginger treatment might also increase the defense mechanism of the body against infections in diabetic rabbits. As mentioned above, it was found that RBC count increased to control level in green tea and/or ginger extract treated diabetic rabbits. Therefore, return of blood indices (MCV, MCH, MCHC) in diabetic treated rabbits could be due to a normalized RBC count in these rabbits. From the hematological results, it is apparent that oral administration of green tea and/or ginger extract might decrease the diabetes-induced disturbances of hematological parameters in alloxan-induced diabetic rabbits.

Data of the present study revealed that daily treatment with green tea and ginger extracts markedly improves hematological, immunobiochemical statuses of rabbits with alloxan-induced diabetes. In conclusion, our study suggests that green tea and ginger extracts if taken together may have more beneficial effects in diabetes as they may have synergistic action, thus holding the hope of a new generation of anti-diabetogenic drugs. However, comprehensive chemical and pharmacological research is required to find out the exact mechanism of green tea and ginger for their antidiabetogenic effect and to identify the active constituent(s) responsible for their effect.

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EFFECTIVENESS OF THE DERYNG AND CLEVENGER-TYPE APPARATUS IN ISOLATION OF VARIOUS TYPES OF COMPONENTS OF ESSENTIAL OIL FROM THE *MUTELINA PURPUREA* THELL. FLOWERS

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Abstract: In this study, both qualitative and quantitative analyses of chemical composition of *M. purpurea* essential oil obtained in the Deryng and Clevenger-type apparatuses were compared. As a result, content of volatile compounds were: 785.67 mg/mL and 833.33 mg/mL in the oil obtained in the Deryng (D-EO) and Clevenger-type apparatuses (C-EO), respectively. The major components of both essential oils from *M. purpurea* were: a-pinene, sabinene, myrcene, (Z)-sesquisabinene hydrate, (E)-sesquisabinene hydrate, and a-bisabolol. The correlation coefficients values are not determined by the differences in the concentrations of the components resulting from the application of two different methods of distillation.

Keywords: essential oil, hydrodistillation, Deryng apparatus, Clevenger-type apparatus, *Mutellina purpurea*

Plant extracts, especially essential oils, have been employed in pharmaceutical, agronomic, food, cosmetic, and perfume industries due to several reported biological properties. From among 3000, approximately 300 isolated oils are commonly used. The main methods to obtain essential oils from the plant materials are steam or water distillation, cold expression or dry distillation. (1-3).

The composition of essential oil (EO), even within one specie, depends on many factors such as growing conditions, time and place of the collection of raw material, drying and storage. The method of determination also has an impact on the qualitative and quantitative analysis of chemical compounds present in the tested EO. Methods for assessing the composition of essential oils can be broadly divided into two research directions. The solid phase microextraction (SPME) method analyzes the composition of the volatile compounds around the plant (4, 5). SPME is a preliminary analysis that requires further action in order to obtain the essential oil in the form that can be applied. The most common method for obtaining the essential oil from the plant material is distillation. Most often it is carried out using glass Clevenger-type or Deryng apparatus. Both devices are recommended pharmacopoeial apparatuses for determining the essential oil content, the former one is described by the European Pharmacopoeia (6) and the latter by the Polish Pharmacopoeia (7).

The volatile composition of the essential oils from different parts of the *Mutelins purpurea* Thell. has been studied earlier (8, 9). However, there is no detailed information about the effect of chosen distillation apparatus on types of isolated compounds. The objective of this study was to compare the effectiveness of Deryng and Clevenger-type apparatus in isolation of various types of components of essential oil from the *Mutelins purpurea* Thell. flowers.

The present research is a continuation of studies comparing essential oils extraction in different

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apparatuses. In an earlier work it was shown that there are differences in the percentage of the chemical composition in the essential oils of sage (10). As the authors found, these differences may result from different times of EO distillation in the Deryng and Clevenger-type apparatuses. In the present paper, the same distillation time was applied to obtain the *M. purpurea* EO in both apparatuses. This could have an impact on the content of the identified compounds, as was evidenced by the high correlation coefficients.

EXPERIMENTAL

Plant material

M. purpurea flowers were collected in the Botanical Garden of the Medical University in Lublin in June 2010. The voucher specimen has been deposited at the Herbarium of the Department of Pharmacognosy, Medical University in Lublin (ES032011M).

Isolation procedure

The fresh plant material (50.0 g) was placed in a round-bottomed flask and 500 mL distilled water was added. Hydrodistillation was performed simultaneously for 3 h by means of the Deryng apparatus and the Clevenger-type apparatus. The obtained oils were dried over anhydrous sodium sulfate and stored at 4°C before the GC analysis. Analyses were repeated three times.

Chemical analysis of essential oil

GC/MS and GC/FID conditions

The gas chromatograph Varian 450-GC with the type triple quadrupole Varian 320-MS was used. The analytes were separated on a 30 m × 0.25 mm VF-5ms capillary column coated with a 0.25 μm film of 5% phenyl methylpolysiloxane, and were inserted directly into the ion source of the MS. The split injection 1 : 100 was used for the samples. The column oven temperature was programmed at 4°C/min from an initial temperature of 50°C (held for 1 min) to 250°C, which was held for 10 min. The injection temperature was 250°C and the injection volume was 1 μL. Helium (99.999%) was used as carrier gas at a flow rate of 0.5 mL/min. The ionizing electron energy was 70 eV and the mass range scanned was 40-1000 m/z with 0.8 s/scan. Manifold temp. was 45°C, transfer line temp. was 289.5°C and the ion source temp. was 271.2°C.

The range of concentrations of constituents outlier in this group GC Varian 3800 (Varian, USA) equipped with a CP-8410 auto-injector and a 30 m × 0.25 mm DB-5 column (J&W Scientific, USA), film thickness 0.25 μm, carrier gas - helium 0.5 mL/min, injector and detector FID temperatures of 260°C; split ratio 1 : 100; injection volume 5 μL. A temperature gradient was applied (50°C for 1 min, then incremented by 4°C/min to 250°C, 250°C for 10 min).

Qualitative and quantitative analysis of essential oils

The qualitative analysis was carried out on the basis of MS spectra, which were compared with the spectra of the NIST/EPA/NIH Mass Spectral Library Search Program (11) and with the data available in the literature (12, 13). The identity of the compounds was confirmed by their retention indices (14), taken from the literature (12, 13) and our own data for standards (α-pinene, *p*-cymene, limonene, γ-terpinene, linalool, (*E*)-caryophyllene, caryophyllene oxide).

The quantitative analysis was performed by means of the internal standard addition method (alkanes C_{12} and C_{19}) according to previously described procedures (15). Essential oil was diluted 1000 times using n-hexane to achieve 1 mL volume, then 1 mg of C_{12}, and 1 mg C_{19} was added into the diluted oil. Samples so prepared were subjected to GC-MS and GC-FID determinations. The quantitative analysis was performed on the basis of calibration curves plotted to find the dependence between the ratio of the peak area for the analyte to the area for internal standard ($A_{analyte} : A_{i.s.}$) *vs*. the analyte concentration ($C_{analyte}$), for α-pinene, *p*-cymene, limonene, γ-terpinene, linalool, (*E*)-caryophyllene, caryophyllene oxide, in the appropriate concentration range (15). The following alkanes were applied as internal standards: C12 (for compounds with retention index < 1300, α-pinene, *p*-cymene, γ-terpinene, linalool); and C19 (for compounds with retention index > 1300, *(E)*-caryophyllene, caryophyllene oxide). The contents of the analyzed substances were read from the achieved calibration curves, the data for which originated from peak areas for *M. purpurea* oil components and internal standard peak areas from GC separation. The final result took into account all dilutions during the whole analytical procedure.

Statistical analysis

All calculations were done using a Statistica 7.1 (StatSoft®, Kraków, Poland) software. Average values were calculated. The results are expressed as the mean ± SD. The chemical composition of the oils was evaluated by Wilcoxon signed-rank test and the sign test.

Table 1. Comparison of the composition of *M. purpurea* flowers essential oil obtained by means of the Deryng and Clevenger's type apparatus.

Compound	RI_{exp}[1]	Concentration in the oil[2] (mg/mL)			
		DERYNG	+/- SD	CLEVENGER	+/- SD
Monoterpenes					
α-thujene	932	1.16	0.25	1.49	0.28
α-pinene	939	76.60	3.40	105.83	2.65
camphene	954	8.58	0.32	11.01	0.16
sabinene	975	189.90	6.33	161.11	4.53
β-pinene	980	13.83	0.96	12.56	0.16
myrcene	989	70.16	1.54	65.76	1.28
limonene	1028	26.26	2.27	30.06	0.59
β-phellandrene	1030	9.76	0.82	4.56	0.12
(*Z*)-b-ocimene	1033	7.58	0.36	8.10	0.18
(*E*)-b-ocimene	1044	18.25	0.26	18.78	0.02
γ-terpinene	1056	10.84	0.11	4.04	0.02
terpinolene	1082	2.41	0.02	1.19	0.10
Total		435.33		424.49	
Oxygenated monotrpenes					
(*Z*)-sabinene hydrate	1068	1.69	0.07	1.40	0.07
linalool	1095	1.00	0.14	1.39	0.00
(*E*) sabinene hydrate	1098	1.11	0.11	1.27	0.00
(*Z*)-p-menth-2-en-1-ol	1122	0.76	0.12	1.05	0.06
terpinen-4-ol	1182	12.89	0.87	6.21	0.48
carvacrol methyl ether	1242	1.88	0.40	1.06	0.02
lavandulyl acetate	1287	1.66	0.05	1.32	0.01
bornyl acetate	1290	4.61	0.20	2.64	0.14
Total		25.60		16.34	
Sesquiterpenes					
β-elemene	1393	19.73	2.84	15.51	0.35
(*E*)-caryophyllene	1427	11.43	0.06	20.28	0.71
γ-elemene	1436	0.28	0.04	0.65	0.13
(*E*)-α-bergamotene	1440	0.12	0.02	0.31	0.01
(Z)-β-farnesene	1460	5.20	0.24	6.03	0.28
α-humulene	1467	1.01	0.16	1.52	0.16
β-acoradiene	1489	0.50	0.16	0.59	0.07
germacrene D	1495	11.50	0.20	20.00	0.93
α-selinene	1503	0.23	0.04	0.31	0.01
bicyclogermacrene	1510	11.75	0.08	13.10	0.60
β-bisabolene	1515	0.38	0.02	0.36	0.04
germacrene A	1521	10.50	0.95	7.18	0.03
δ-amorphene	1531	1.15	0.15	1.10	0.05
β-sesquiphellandrene	1554	0.74	0.06	0.77	0.04
germacrene B	1571	6.89	1.24	10.06	0.21
viridiflorene	1605	0.20	0.03	0.53	0.03
Total		81.61		98.30	

Table 1. cont.

Compound	RI_{exp} [1]	Concentration in the oil [2] (mg/mL)			
		DERYNG	+/- SD	CLEVENGER	+/- SD
Oxygenated sesquiterpenes					
sesquicineole	1525	1.04	0.42	1.32	0.10
(Z)-sesquisabinene hydrate	1564	54.59	1.38	63.06	0.93
spathulenol	1588	6.73	0.07	12.10	0.16
caryophyllene oxide	1593	0.87	0.07	2.91	0.14
(E)-sesquisabinene hydrate	1598	67.76	1.13	75.79	1.55
β-atlantol	1624	1.03	0.17	1.34	0.02
1-*epi*-cubenol	1638	1.80	0.18	1.72	0.01
α-acorenol	1647	8.79	0.49	8.80	0.22
epi-α-muurolol	1658	1.46	0.61	1.88	0.07
β-acorenol	1666	2.39	0.14	2.23	0.06
α-cadinol	1671	4.34	0.17	5.10	0.03
neo-intermedeol	1675	1.54	0.20	1.50	0.04
β-bisabolol	1685	12.42	0.39	12.16	0.32
bulnesol	1691	0.90	0.10	1.30	0.10
α-bisabolol	1703	37.20	1.17	56.59	0.62
(Z)-farnesol	1712	6.97	0.55	8.09	0.27
(Z)-α-bisabolene epoxide	1726	0.23	0.04	0.75	0.01
Total		210.06		256.64	
Aromatic compounds					
p-cymene	1024	16.87	0.75	15.34	0.26
m-cresol	1071	1.34	0.57	2.26	0.08
methyl eugenol	1404	1.38	0.18	1.49	0.22
2.5-dimethoxy-*p*-cymene	1418	1.30	0.10	2.19	0.04
Total		20.89		21.28	

[1] – retention time on the column VF - 5 ms. [2] – concentration of the compound in the sample (mg) on the basis of the internal standard comparison.

RESULTS AND DISCUSSION

The obtained content of volatile compounds were: 785.67 mg/mL and 833.33 mg/mL in the oil obtained in the Deryng (D-EO) and Clevenger-type apparatuses (C-EO), respectively. In both essential oils 58 identical compounds were identified. The quantitative analysis revealed differences between the methods. Table 1 shows the qualitative and quantitative comparison of the components of the essential oils obtained by means of two methods.

Composition of essential oils obtained in Deryng and Clevenger-type apparatuses

The dominant components of D-EO were sabinene > α-pinene > myrcene > *(E)*-sesquisabinene hydrate > *(Z)*-sesquisabinene hydrate > α-bisabolol. In the case of C-EO the sequence was similar. Only myrcene content differed quantitatively. The structures and the quantitative comparison of the major essential oils components are shown in Figs. 1 and 2.

Analyzing the groups of chemical compounds present in the oils obtained in both apparatuses, it should be noted that in both cases monoterpenes were predominant: D-EO 55.4% (435.33 mg/mL), and C-EO 50.1% (424.49 mg/mL). The lowest amount was recorded for oxygenated monoterpenes: D-EO 3.3% (25.60 mg/mL), and C-EO 2.0% (16.34 mg/mL).

A high amount of sabinene and α-pinene in the essential oils is characteristic of Apiaceae family

(16). The dominant components were sabinene and α-pinene. In our study, sabinene and α-pinene, content were 189.9 and 76.60 mg/mL, respectively, in the Deryng, and 161.11 and 105.83 mg/mL, respectively, in the Clevenger-type apparatus, confirming the literature data on the chemotaxonomic characteristics of Apiaceae family.

According to the European Pharmacopoeia (6) and the Polish Pharmacopoeia VI (7) an essential oil is the plant extract obtained just by distillation processes, like hydrodistillation, with the exception of the *Citrus* sp. peel oil, which is isolated by cold expression. When other isolation techniques are employed, other designations, such as volatiles or

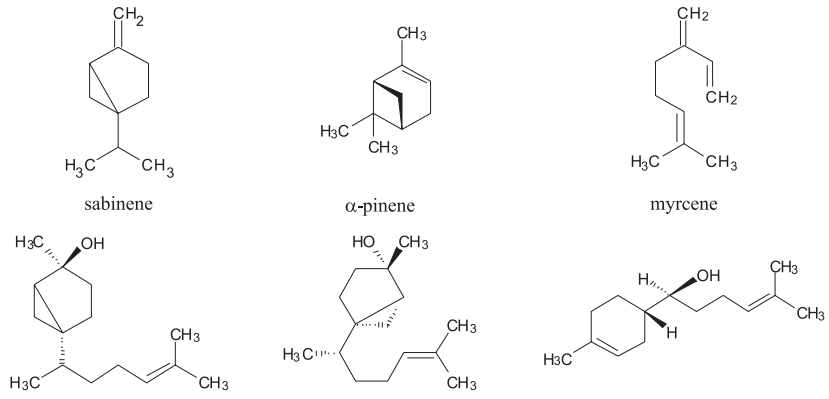

Figure 1. Structures of main compounds of essential oils of *M. purpurea* flowers

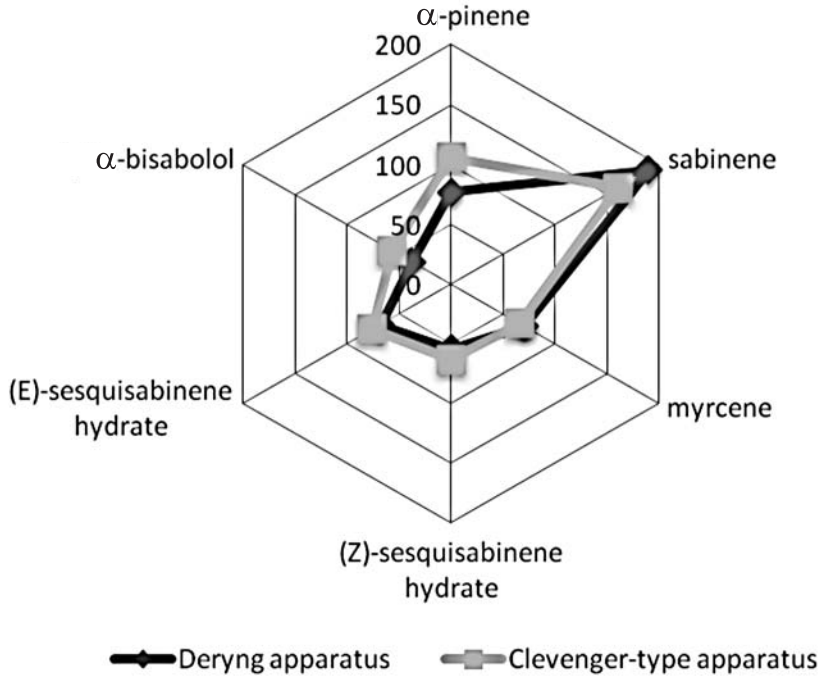

Figure 2. Comparison of the main components of essential oils from *M. purpurea* received by means of the Deryng and Clevenger-type apparatus

volatile oil, must be used (17). Hydrodistillation is a conventional method used to extract essential oils from aromatic plants. It can be used in industry and gives no chemical pollutions (18). The essential oil is obtained using the equipment usually based on the circulatory distillation approach using Deryng and Clevenger-type apparatuses. In theory, the recoveries of volatiles are quantitative for an infinite distillation time; thermal artifacts can be produced but they are accepted as a result of the traditional process.

Most hydrodistillation methods used in order to standardize essential oils apply a Clevenger-type apparatus as a Pharmacopoeial apparatus. Alternatively, a Deryng apparatus can be used. This device was included for many years in the Polish Pharmacopoeia and formed the basis of the qualitative evaluation of aromatic plant materials for use in the pharmaceutical industry. So far, it has been still used in many laboratories. Its compact design makes it even more convenient to use, whether to modify hydrodistillation methods or for the pre-screening of raw aromatic materials.

The qualitative analysis showed that the essential oils obtained in both apparatuses did not differ in the number of chemical compounds present. The quantitative differences between the main components were small.

Statistical analysis

The results presented in Table 1 were submitted to statistical analysis using tests assessing the significance of the difference between two dependent samples, which were essential oils obtained from the same plant material by hydrodistillation in the Deryng and the Clevenger-type apparatuses.

A null hypothesis was formed that there was no difference between the results of quantitative and qualitative analyses of oils distilled by means of those two different methods. The sign test showed that the calculated significance level $p = 0.0256$ was lower than the accepted one ($p = 0.05$), thus the hypothesis was rejected. Wilcoxon test confirmed those results and calculated the significance level also below 0.05 and it amounted to 0.0454. The results of both tests indicate that the quantitative and qualitative composition of essential oils obtained from the same raw material is dependent on the method by means of which it was obtained. Therefore, there is no basis to determine which type of apparatus is more suitable for oil extraction.

As shown in Fig. 3, the components found in the oil in the highest quantity are as follows: α-pinene, sabinene, myrcene, *(Z)*-sesquisabinene hydrate, *(E)*-sesquisabinene hydrate and α-bisabolol.

Despite the fact that the composition of essential oils acquired from the same raw material is

Table 2. The values of correlation coefficients and their interpretation (n = number of components).

Group of volatile compounds	Correlation	Range of constituents in similar quantities	Range of concentrations of constituents outlier in this group
Monoterpenes (n = 15)	0.9732	1.16 - 30.06 (n = 12)	65.76 - 189.90 (n = 3) sabinene, α-pinene, myrcene
Oxygenated monoterpenes (n = 8)	0.9935	0.76 - 4.61 (n = 7)	6.21 - 12.89 (n = 1) terpinen-4-ol
Sesquiterpenes (n = 16)	0.8878	0.12 - 1.52 (n = 9)	5.20 - 20.28 (n = 7) β-elemene, (*E*)-caryophyllene, (*Z*)-β-farnesene, germacrene D, bicyclogermecrene, germacrene A, germacrene B
Oxygenated sesquiterpenes (n = 17)	0.9891	0.23 - 12.42 (n = 14)	37.20 - 75.79 (n = 3) (*Z*)-sesquisabinene hydrate, (*E*)-sesquisabinene hydrate, α-bisabolol
Aromatic compounds (n = 4)	0.9985	1.30 - 2.26 (n = 3)	15.34 - 16.87 (n = 1) p-cymene

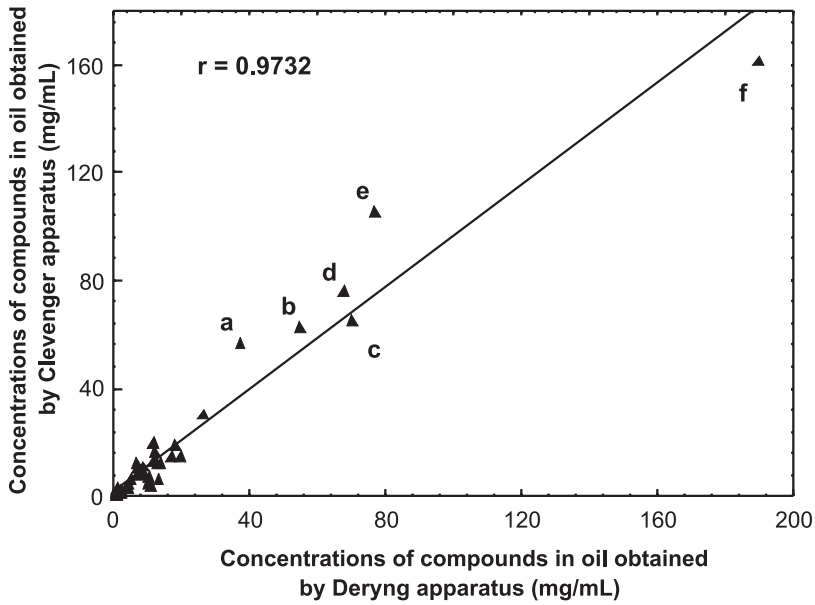

Figure 3. Correlation between the content of compounds of essential oils obtained in the Deryng and Clevenger-type apparatus from *M. purpurea* flowers. (a) α-bisabolol, (b) (*Z*)-sesquisabinene hydrate, (c) myrcene, (d) (*E*)-sesquisabinene hydrate, (e) α-pinene, (f) sabinene

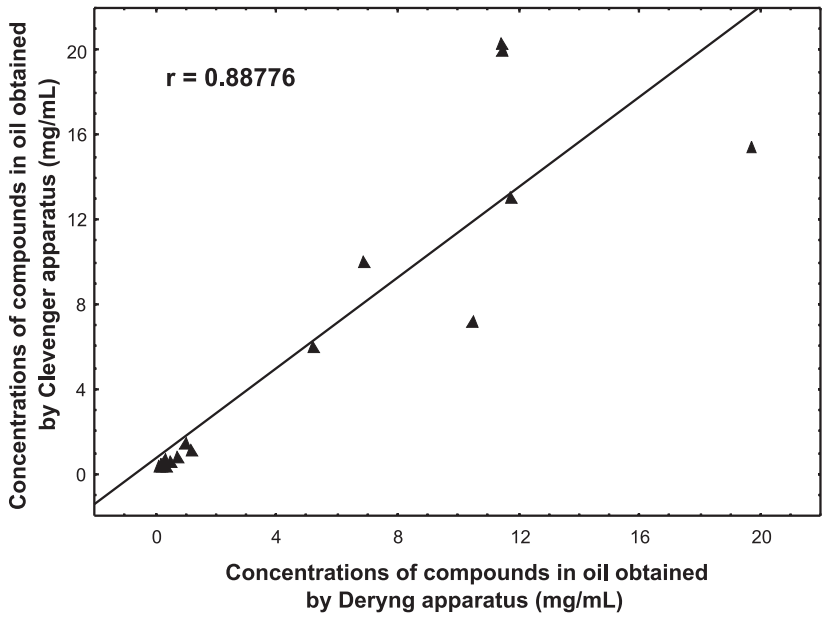

Figure 4. Correlation between the content of sesquiterpenes obtained in the Deryng and Clevenger-type apparatus from *M. purpurea* flowers

dependent on the distillation method, a high value of the correlation coefficient (r = 0.9732) can be found between the concentrations of the compounds in both oils. The following equation y = 1.4794 + 0.9515 x describes this relationship. It suggests that both groups of results are highly correlated and only a few components of the oils differ significantly in concentration.

The next step of comparing the composition of the essential oil obtained in both apparatuses was

determination of the relationship between the groups of identified compounds.

Table 2 shows the relationship between the groups of components of essential oils obtained during the distillation process in the both apparatuses.

The linear relationship for all groups presents a very similar picture: a large number of components grouped in the lowest concentration range, then a cluster with the highest concentration of one or three components. For sesquiterpenes, seven components with higher concentrations are grouped in a wide range of concentrations (see Fig. 4).

The highest correlation coefficients were obtained for the following two groups: aromatic compounds (n = 4, r = 0.9985) and oxygenated monoterpenes (n = 8, r = 0.9935). However, both groups are characterized by two features:

- a small number of components,
- one component at a concentration exceeding several times the average concentration of the other ingredients in the group.

In this situation, the differences in the concentrations of other constituents resulting from the application of two different methods of distillation have a negligible effect on the value of the correlation coefficients. They have very small values comparing to the component with an extreme concentration value.

Slightly lower values of correlation coefficients were obtained for the following two groups: oxygenated sesquiterpenes (n = 17, r = 0.9891) and monoterpenes (n = 15, r = 0.9732). They contain three ingredients in terms of concentrations, from a few to several times exceeding the average concentration of the other ingredients in the group. Three components which give direction of the correlation reduce its value.

The lowest value of the correlation coefficient was obtained for sesquiterpenes (n = 16, r = 0.8878). Regardless of the method of the oil production, the concentrations of 9 components are located in a narrow range of values, thus creating the initial section of the linear relationship. The direction of the linear relationship gives 7 other components present in a wide concentration range from a few to several times exceeding the average concentration of the other ingredients in the group. The result of a lack of a single component at a high concentration comparing to the other ingredients is that the direction of the correlation is given by a few components. The dispersion of their concentrations reduces the value of the correlation coefficient.

The correlation coefficients values are not determined by the differences in the concentrations of the components resulting from the application of two different methods of distillation. They are determined by the individual components at concentrations much higher than the average concentration of the other ingredients in the group. They give the correlation direction, and the fewer such components in the group, the higher the value of the correlation coefficient is achieved.

The present work is a detailed analysis of the essential oils content and it reflects the impact of the construction of the apparatus on the composition of the essential oil, but the differences can be acceptable for future biological research. Hydrodistillation in the Deryng and Clevenger-type apparatuses yielded 10.09 mL/kg and 16.80 mL/kg (dry weight) of essential oil, respectively. Applying the same conditions it was possible to obtain 60.1% more essential oil in a Clevenger apparatus. Analyzing the quantitative composition of both essential oils it cannot be said that the differences between individual compounds were proportional. The oil obtained in a Deryng appaeatus was abundant in monoterpenes whereas the dominant group in a Clevenger-type apparatus were sesquiterpenes. The statistical analysis suggests that both groups of essentials oils are highly correlated and only a few components of the oils differ significantly in concentration.

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APPLICATION OF CHEMOMETRICS FOR IDENTIFICATION OF PSYCHOACTIVE PLANTS

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Abstract: Drug market changes dynamically causing many analytical challenges for police experts. Among illicit substances there are synthetic designer products but also herbal material. Plant material is usually in fine-cut or powdered form, thus difficult to identify. For such fragmented material classic taxonomical identification methods using anatomical and morphological features of the plant cannot be employed. The aim of the study was to develop an identification method of the powdered material with employment of multidimensional data analysis techniques. Principal Component Analysis (PCA) was chosen as a method of data exploration. The study was conducted on four plants controlled in Poland: *Salvia divinorum*, *Mitragyna speciosa*, *Psychotria viridis* and *Calea zacatechichi*. The compatibility of grouping features of selected species was compared in two variants: chemical and elemental composition. In a first variant, GC-MS chromatograms of extracts were analyzed and in the second, elements composition with the AAS and the ICP-MS techniques. The GC-MS method, based on the qualitative interpretation of results, allows for clear differentiation of samples with regard to their species affiliation. Even the plants belonging to the same family *Rubiaceae*, *P. viridis* and *M. speciosa* formed homogeneous and clearly separated clusters. Additionally, the cluster analysis was performed, as a method confirming sample grouping.

Keywords: narcotic plants, chemometrics, identification, gas chromatography, elements

Narcotics available on illegal market often contain psychoactive plant material. In many cases this material derives from species illegal in particular country. Plant material is usually in fine-cut or powdered form, thus difficult to identify. For such fragmented material classic taxonomical identification methods using anatomical and morphological features of the plant cannot be employed (1). For plants, in which psychoactive compound is species-specific, identification includes botanical and phytochemical analysis. Identity of *Salvia divinorum* Epling et Játiva-M. (*Lamiaceae*) and *Mitragyna speciosa* Korthals (*Rubiaceae*) can be confirmed by detection of secondary metabolites, salvinorin A and mitragynine, respectively (2). In a case where the psychoactive compound is not specific to the particular species, such as N,N-dimethyltryptamine for *Psychotria viridis* Ruíz et Pavón (*Rubiaceae*) (3), unambiguous determination whether a test material is derived from an illegal plant, may be problematic. The biggest problem, however, like in case of *Calea zacatechichi* Schlechtendal (*Asteraceae*), is the identification of the plant, in which no psychoactive substance or the other marker was found (4).

The aim of the study is to develop a method enabling for identification of plant species controlled according to Polish law, based on their chemical characteristics without the need for determination of specific compounds. Chemometric techniques have such potential. They allow building a classification model, in which objects are grouped according to their taxonomic assignment of the species. The data on which the model is constructed derived from the characteristics of the plant. The features used in this technique may consist of micromorphological (5, 6), chemical (7-9) or elemental composition of plant (10). Identification of species using chemometric techniques is becoming more and more popular, but so far it has not been used for the assessment of illegal plant products.

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Table 1. Mineralization parameters of sample preparation for ASA and ICP-MS analyses.

Step	Time of heating (s)	Time of cooling (s)	Microwave power (W)
1	120	120	87.5
2	120	600	157.5
3	120	120	210.0
4	120	600	245.0
5	120	120	280.0
6	300	600	315.0

Table 2. Conditions of the F-AAS method.

Element	Flame type	Analyte wavelength [nm]	Internal standard	Working range [mg/L]
Ca	Nitrous oxide/acetylene	422.7	yes	0.25 – 1.25
Fe	Air/acetylene	248.3	no	0.5 – 6
K	Air/acetylene	766.5	no	0.1 – 1.0
Mg	Air/acetylene	285.2	yes	0.1 – 0.5
Na	Air/acetylene	589.0	no	0.25 – 1.0
Zn	Air/acetylene	213.9	no	0.1 – 1.0

Table 3. Eigenvalues of the principal components obtained from GC-MS results.

	Eigenvalues	% of the total variance	Accumulated eigenvalues	Accum. - % of the total variance
1	52.26088	18.02099	52.2609	18.0210
2	43.31437	14.93599	95.5752	32.9570
3	32.18627	11.09871	127.7615	44.0557
4	22.84326	7.87699	150.6048	51.9327
5	16.63155	5.73502	167.2363	57.6677
6	15.22790	5.25100	182.4642	62.9187
7	14.41694	4.97136	196.8812	67.8901
8	13.27539	4.57772	210.1566	72.4678
9	12.03532	4.15011	222.1919	76.6179
10	11.25389	3.88065	233.4458	80.4985
11	9.38060	3.23469	242.8264	83.7332
12	8.02784	2.76822	250.8542	86.5015
13	7.24924	2.49974	258.1034	89.0012
14	7.11811	2.45452	265.2216	91.4557
15	5.60138	1.93151	270.8229	93.3872
16	4.69090	1.61755	275.5138	95.0048
17	3.97270	1.36990	279.4865	96.3747
18	3.68404	1.27036	283.1706	97.6450
19	3.36614	1.16074	286.5367	98.8058
20	2.18997	0.75516	288.7267	99.5609
21	1.27333	0.43908	290.0000	100.0000

The specific aims of this study is to use the chemometric techniques to identify narcotic plants in their powdered form. The study was conducted on four plants controlled in Poland: *S. divinorum*, *M. speciosa*, *P. viridis* and *C. zacatechichi*. We compared the compatibility of grouping features of selected species in two variants: chemical and elemental composition. In a first variant, GC-MS chromatograms of extracts were analyzed and in the second, elements composition using AAS (Ca, Fe, K, Mg, Na, Zn) and the ICP-MS techniques (Ag, Al, B, Ba, Be, Cd, Ce, Co, Cr, Cs, Cu, Dy, Eu, Ga, Gd, Hg, Li, Mn, Mo, Nd, Ni, Pb, Pr, Rb, Sb, Sm, Sr, U, V). Choosing the right test, with data characterizing the material according to the taxonomic assignment of the species, distinct clusters can be obtained. The optimal grouping allows for the construction of the classification model as a new tool for plant material identification in forensic laboratories.

EXPERIMENTAL

Plant materials

The examined products were purchased from the Internet. According to the provider's declaration the investigated material was: dried shredded leaves of *Salvia divinorum* from Mexico - four samples S; dried leaves of *Psychotria viridis* from Hawaii - sample P(H), two samples from Brazil – P(B) and three samples from Peru – P(P); dried shredded or powdered leaves of *Mitragyna speciosa* from Thailand – three samples M(T) and three samples from Bali – M(B); and dried shredded herb of *Calea zacatechichi* from Mexico – five samples C.

Reagents and materials

Methanol, chloroform, ethyl acetate, dichloromethane and hexane pure p.a. were purchased from POCH (Poland). Deionized water was obtained with

Table 4. PC1. PC2 and PC3 values obtained from GC-MS results corresponding to the plant samples: S – *S. divinorum*; P(H) – *P. viridis* from Hawaii; P(B) – *P. viridis* from Brazil; P(P) – *P. viridis* from Peru; M(T) – *M. speciosa* from Thailand; M(B) – *M. speciosa* from Bali and C – *C. zacatechichi*.

Sample	PC1	PC2	PC3
S	0.4738	1.9206	0.3141
S	0.4596	1.8023	0.3572
S	0.5656	1.9082	0.2824
S	0.4685	2.2157	0.5758
P(H)	0.4896	-0.5476	-1.2652
P(B)	0.4669	-0.4627	-1.1980
P(B)	0.4532	-0.4247	-1.1696
P(P)	0.4964	-0.0875	-1.4251
P(P)	0.5246	-0.1882	-1.1493
P(P)	0.4716	-0.3891	-1.2706
P(P)	0.4170	-0.3511	-1.5186
M(T)	0.6054	-0.8545	1.2147
M(T)	0.5585	-0.6953	1.0609
M(T)	0.6610	-0.8655	1.1713
M(B)	0.5683	-0.8936	1.1969
M(B)	0.6053	-0.8676	1.1051
M(B)	0.6461	-0.9753	1.3668
C	-2.1012	-0.0014	0.3661
C	-1.8771	-0.1466	0.1522
C	-1.9034	0.0014	0.0370
C	-1.5961	-0.1167	-0.0250
C	-1.4535	0.0192	-0.1790

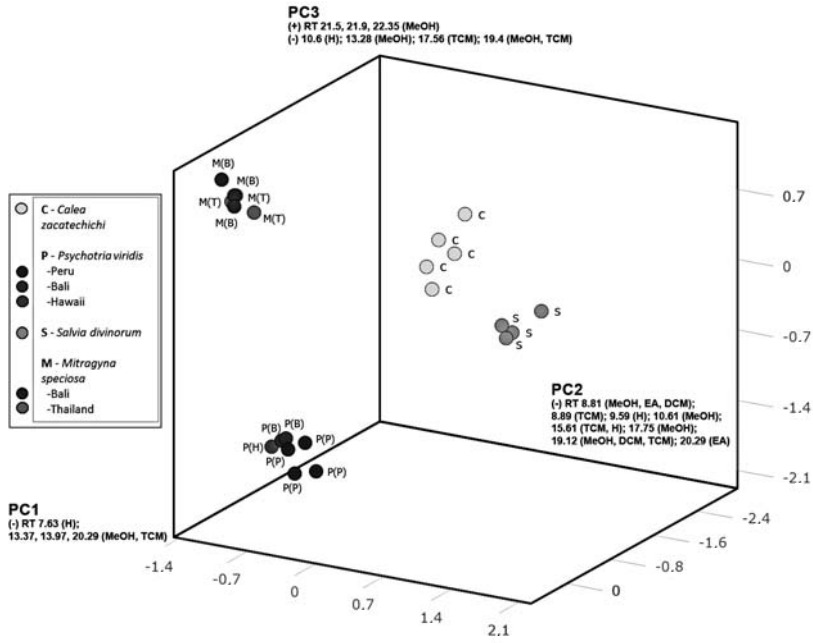

Figure 1. PC1, PC2 and PC3 scores for GC-MS results according to the species: S – *S. divinorum*; P(H) – *P. viridis* from Hawaii; P(B) – *P. viridis* from Brazil; P(P) – *P. viridis* from Peru; M(T) – *M. speciosa* from Thailand; M(B) – *M. speciosa* from Bali and C – *C. zacatechichi*

Table 5. Eigenvalues of the principal components obtained from elemental composition.

	Eigenvalues	% of the total variance	Accumulated eigenvalues	Accum. - % of the total variance
1	10.67761	30.50745	10.67761	30.5075
2	5.66157	16.17591	16.33918	46.6834
3	3.88256	11.09304	20.22174	57.7764
4	3.75177	10.71934	23.97351	68.4957
5	2.50624	7.16069	26.47975	75.6564
6	2.35411	6.72603	28.83386	82.3825
7	1.24418	3.55479	30.07804	85.9373
8	1.15646	3.30418	31.23450	89.2414
9	0.89630	2.56085	32.13080	91.8023
10	0.82497	2.35705	32.95577	94.1593
11	0.50425	1.44071	33.46002	95.6000
12	0.40901	1.16861	33.86903	96.7686
13	0.34960	0.99886	34.21863	97.7675
14	0.26917	0.76906	34.48780	98.5366
15	0.20557	0.58735	34.69337	99.1239
16	0.10717	0.30621	34.80054	99.4301
17	0.08818	0.25195	34.88873	99.6821
18	0.05438	0.15537	34.94311	99.8375
19	0.02698	0.07707	34.97008	99.9145
20	0.01904	0.05440	34.98913	99.9689
21	0.01087	0.03107	35.00000	100.0000

deionizating filter system Water PRO PS from Labanco (Kansas City, USA). Membrane filters Chromafil Pet-45/25 from Macherey-Nagel GmbH & Co.KG (Germany).

ICP-MS and AAS reagents: stock solution IV-ICPMS-71A: Ag, Al, As, B, Ba, Be, Cd, Ce, Co, Cr, Cs, Cu, Dy, Eu, Ga, Gd, Mn, Nd, Ni, Pb, Pr, Rb, Se, Sm, Sr, Tl, U, V in 10.00 µg/mL from Inorganic Ventures (USA); standard solution of: Ca, Fe, Hg, In, K, Li, Mg, Mo, Na, Sb, Zn in 1000 µg/mL from Merck (Germany); nitric acid of ICP grade from Merck (Germany); lanthanum nitrate of AAS grade from Merck (Germany); redistilled water with Nanopure Diamond UV system from Barnstead. Argon 99,999%, nitrous oxide and acetylene min. 99,6% from Air Products.

Apparatus

GC-MS Agilent GC7890A+5975C VLMSD instrument from Agilent Technologies (USA); ICP-MS spectrometer Thermo Electron X Series II from Thermo Electron Corporation (USA); AAS Solaar M6 from Thermo Elemental; hollow cathode lamps: Ca-HCL, Fe-HCL, K-HCL, Mg-HCL, Na-HCL and Zn-HCL; microwave decomposition unit UniClever from Plazmatronika-Service (Poland); vibratory grinder with mill made of corundum from Testchem (Poland) were used

Sample preparation

For GC-MS analysis, powdered dried plant material (0.5 g) was extracted with 5.0 mL of methanol (MeOH), ethyl acetate (EA), dichloromethane (DCM), chloroform (TCM) and hexane (H) in an ultrasound bath for 30 min and filtered through membrane filters.

For ICP-MS and AAS methods, dried plant material was ground in vibratory grinder and the lost of drying in 105°C was determined. One gram sample weights were placed in Teflon crucibles and 5 mL of nitric acid was added. The digestion using microwave energy in a closed system was performed in a six-step system. Mineralized solutions were transferred to volumetric flasks and filled up to 100.0 mL with water. Mineralization parameters are presented in Table 1.

Methods

GC-MS

GC-MS was performed with a 5% phenylmethylpolysiloxane capillary column HP-5MS (30 m × 0.25 mm i.d.; film thickness, 0.25 µm; Agilent J&W Scientific (USA). The temperature program consisted

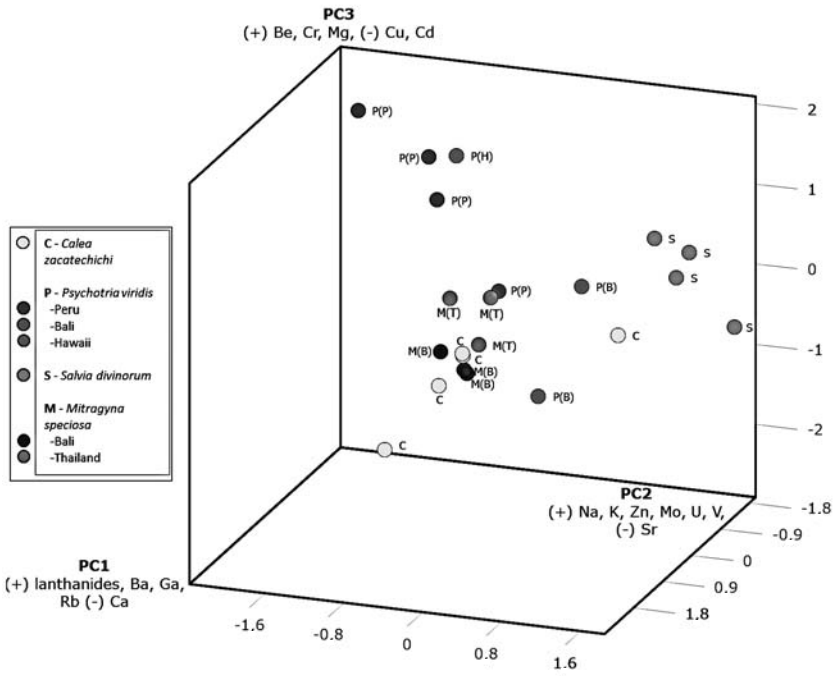

Figure 2. PC1, PC2 and PC3 scores for elemental composition according to the species: S – *S. divinorum*; P(H) – *P. viridis* from Hawaii; P(B) – *P. viridis* from Brazil; P(P) – *P. viridis* from Peru; M(T) – *M. speciosa* from Thailand; M(B) – *M. speciosa* from Bali and C – *C. zacatechich*

of the initial temperature of 100°C held for 0.5 min, followed by a linear ramp up to 300°C at 12°C/min. The inlet temperature was set at 230°C, and the injection was set in the split 1:80 mode. The carrier gas was high-purity helium at a flow rate of 1.0 mL/min.

The MS detector parameters used were: interface temperature, 290°C; ion-source temperature, 230°C; ionization mode, electron ionization (EI); ionization voltage, 70 eV; scan time, 1.0 s/scan; scan range, *m*/*z* 40–550.

ICP-MS

The ICP-MS measurement were performed using the following parameters: R.f. power 1302 W, background line < 0.5 counts/s; gas flow: plasma: 12.4–13,3 L/min; support: 0.83–0.89 L/min, nebulizer: 1.08–1.18 L/min, proportion of double charged ions $^{137}Ba^{2+}/^{137}Ba$ less than 3.0%, proportion of oxide ions $^{156}CeO/^{140}Ce$ less than 3.0%; $^{115}In/^{220}Bkg$ > 800 000; sample aspiration time 30 s; acquisition time for 3 replicates 15 s. The indium solution in 10 µg/L concentration was used as an internal standard.

Content of Ag, Al, B, Ba, Be, Cd, Ce, Co, Cr, Cs, Cu, Dy, Eu, Ga, Gd, Hg, Li, Mn, Mo, Nd, Ni, Pb, Pr, Rb, Sb, Sm, Sr, U and V has been determined. The measuring range for the elements was 0.5 – 50.0 µg/L.

F-AAS

Content of Ca, Fe, K, Mg, Na and Zn has been measured. The F-AAS method conditions are placed in Table 2.

One milliliter of 5% lanthanum solution was used as an internal standard. The assay of elements was counted on the dry mass of the plant material.

Statistical analysis

For the statistical analysis "Statistica 10" software from StatSoft (USA) was used, all the other calculations were performed in Microsoft Excel.

Table 6. PC1. PC2 and PC3 values obtained from elemental composition corresponding to the plant samples: S – *S. divinorum*; P(H) – *P. viridis* from Hawaii; P(B) – *P. viridis* from Brazil; P(P) – *P. viridis* from Peru; M(T) – *M. speciosa* from Thailand; M(B) – *M. speciosa* from Bali and C – *C. zacatechichi*.

Sample	PC1	PC2	PC3
S	-1.1468	1.0936	0.5144
S	-1.1086	1.4631	0.4082
S	-1.3274	1.8459	-0.5409
S	-1.2050	1.2998	0.0344
P(H)	-0.9381	-0.8459	1.3401
P(B)	1.8894	1.3483	1.0585
P(B)	2.6989	1.1741	-0.0346
P(P)	-0.3715	-0.9399	1.5147
P(P)	0.1871	-0.6727	1.2216
P(P)	-0.1158	-1.5759	2.0946
P(P)	0.2251	-0.0351	0.1863
M(T)	0.5232	-0.0250	0.2139
M(T)	0.2744	-0.2257	-0.4863
M(T)	0.2249	-0.5309	0.0276
M(B)	0.7183	-0.1982	-0.6780
M(B)	-0.1056	-0.7336	-0.7860
M(B)	1.1548	-0.0871	-0.4535
C	-0.3838	0.9778	-0.4295
C	-0.4244	-0.6130	-0.9323
C	0.2709	-1.1794	-1.9426
C	-0.3348	-0.8274	-1.3057
C	-0.7050	-0.7127	-1.0249

The Principal Component Analysis was carried out in two variants, on GS-MS fingerprints obtained from the plant extracts and on the elemental composition of the plants. GC-MS results formed a data matrix composed on 290 variables and 22 cases. In case of the elements assay, the data matrix was composed on 35 variables and 22 cases.

Using PCA algorithm for calculations, the consistent grouping of objects, in both cases, has been achieved and no rotation has been applied.

RESULTS AND DISCUSSION

Species identification of illegal fragmented plant material is a challenging task. Taxonomic research based only on part of the plant is impossible to be performed. Therefore, it seems that the only effective method of identification may be the use of micromorphological examination combined with phytochemical analysis (11). If the substance responsible for psychoactive activity of the plant is known, especially if it is a specific compound, the species identity of the material can be established. Two plants, *S. divinorum* and *M. speciosa* contain active markers, salvinorin A and mitragynine, respectively. In case of *P. viridis*, the psychoactive compound, DMT, is not specific to the species and does not allow the material identification. In *C. zacatechichi*, no psychoactive substance has been recognized so far, and no specific compounds have been isolated.

The aim of the study was to develop an identification method of the powdered material with employment of multidimensional data analysis techniques. Principal component analysis (PCA) was chosen as a method of data exploration. It is a procedure that uses orthogonal transformation of observable variables into a new set of uncorrelated variables (called components). PCA does not involve variable reduction, and the total variance of variables is equal to the sum of variances of principal components (12). Although it would be worthwhile to use the cluster analysis (CA) for variable grouping, our final aim is that the suggested solution from the principal component space be complemented with subsequent cases in the future. The cluster analysis was performed additionally, as a method confirming sample grouping.

A GC-MS studies of the following extracts were performed: methanol, ethyl acetate, dichloromethane, chloroform and hexane, obtained from *S. divinorum*, *P. viridis*, *M. speciosa* and *C. zacatechichi*. Ten retention times corresponding to the largest peak areas were selected from each chromatogram. Each peak represented a single compound corresponding to mass. Retention times of all the peaks were introduced as variables. The sample was characterized on the basis of the presence or absence of a given compound in the extract. As a result, a matrix having 290 columns and 22 rows containing discrete data was obtained. In the examined set, numerous statistically significant correlations at the significance level of $p = 0.001$ were observed. In order to interpret the results, the principal component analysis was performed. Ten principal components (1-10, Table 3) describing 80% of the total number of variances were used for further calculations. The number of principal components for interpretation was selected on the basis of the Cattell's criterion (13), with the assumption that 20% of the given data variance may be omitted with no difference to significant information contained in the data. Principal component: PC1, PC2 and PC3 values obtained from GC-MS results corresponding to each of the plant sample are presented in Table 4.

On the basis of the analysis of certain factor scores value, it was found out that already three first components allow effective differentiation between samples of various species. The first principal component PC1 allows isolation of *C. zacatechichi* samples, the second component PC2 species *S. divinorum* and the third component PC3 differentiates between the species *M. speciosa* and *P. viridis* (Fig. 1).

The PCA of the element composition was performed on the basis of the content of the following metals in the samples: Ag, Al, B, Ba, Be, Cd, Ce, Co, Cr, Cs, Cu, Dy, Eu, Ga, Gd, Hg, Li, Mn, Mo, Nd, Ni, Pb, Pr, Rb, Sb, Sm, Sr, U and V. In the examined set, numerous statistically significant correlations at the significance level of $p = 0.001$ were observed. In order to interpret the results, the principal component analysis preceded by data standardization was performed. Eight principal components (1-8, Table 5) describing 90% of the total number of variances and meeting the Kaiser criterion were used for further calculations (14). Principal component: PC1, PC2 and PC3 values obtained from elemental composition corresponding to each of the plant sample are presented in Table 6.

The first principal component PC1 differentiates between *S. divinorum* samples and *P. viridis* coming from Brazil (Fig. 2). The species *S. divinorum* and *P. viridis* from Hawaii revealed small amount of lanthanides and a relatively high calcium level, whereas a high level of lanthanides in *P. viridis* samples from Brazil clearly grouped these objects in a separate cluster. Thus, within one *P.*

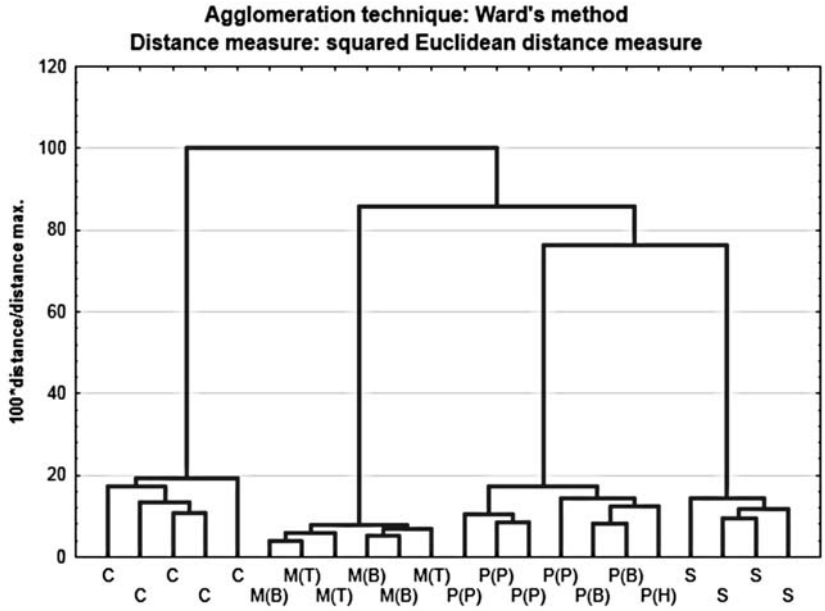

Figure 3. Dendrograms for hierarchical clustering of chemical variables according to the GC-MS chromatograms

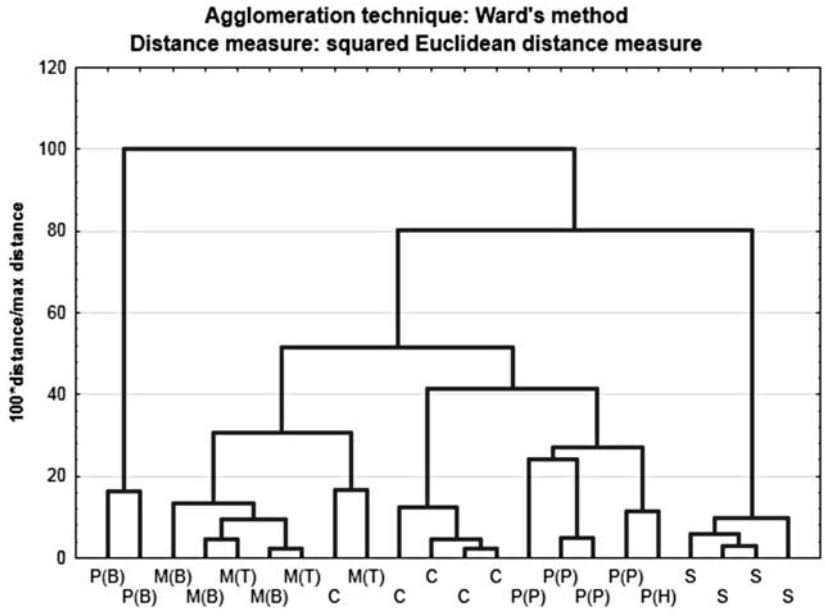

Figure 4. Dendrograms for hierarchical clustering of elemental composition

viridis species, two completely different levels of these metals were observed. The second principal component PC2 differentiates a group comprising *S. divinorum* and *P. viridis* from Brazil *versus P viridis* from Hawaii and Peru and *C. zacatechichi*. *S. divinorum* and *P. viridis* samples from Brazil, in contrast to the second group, contain a larger amount of Na, K, Zn, Mo, U and V, and small amounts of Sr (Fig. 2). The third principal component, PC3 allows isolation of the *P. viridis* group on the basis of a higher

content of Be, Mg, Cr and a low content of Cu and Cd. This component differentiates between *P. viridis* and *C. zacatechichi* (Fig. 2).

The aim of the work was to assess the possibility of using the ICP-MS and GC-MS methods combined with a chemometric analysis for identification of plant narcotics. Sample grouping agreeing with the species affiliation was observed for the results of the GC-MS analysis. Due to low availability of the material, caused by its illegal status, it was impossible to accomplish a model, but the obtained results confirmed the usefulness of PCA for the narcotic plant recognition.

PCA of the results obtained from the ICP-MS and AAS analysis did not lead to formation of clusters representing a single species. This results from the fact that the content of elements in a plant depends on numerous factors. They involve the type of soil and environmental conditions, such as precipitation. In case of endemic plants such as *S. divinorum*, the elemental composition of all the samples was the same. The biggest differences in elements content were observed in *P. viridis*, since the samples came from different places: Hawaii, Brazil and Peru.

The GC-MS method, based on the qualitative interpretation of results, allows for clear differentiation of samples with regard to their species affiliation. Even the plants belonging to the same family - *P. viridis* and *M. speciosa*, formed homogeneous and clearly separated clusters. HCA results presented in dendrograms confirmed that the study of chemical composition is more effective for grouping than the study of elemental composition (Figs. 3 and 4).

CONCLUSIONS

Object grouping, and ultimately a classification model based on the analysis of principal components may be used as a tool for identification of plant narcotics. The method of statistical interpretation of results obtained in the GC-MS analysis of plant-derived extracts presented in this study is an alternative for complex identification procedures involving microscopic methods and phytochemical analysis. Creating a model based on the elemental composition seems to be pointless, due to dependence of the element content of the plant on environmental conditions. However, such a model may be useful for determining the place of origin of the plant material.

The results of this study are an important start in creating a model allowing identification of selected narcotic plants, but they have to be supported with more samples. A good solution may be to initiate cooperation between forensic laboratories in order to provide data and then to use methods based on the chemometric analysis.

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PHARMACEUTICAL TECHNOLOGY

PHARMACOKINETICS OF DICLOFENAC SODIUM AND PAPAVERINE HYDROCHLORIDE AFTER ORAL ADMINISTRATION OF TABLETS TO RABBITS

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Abstract: Non-compartmental pharmacokinetic analysis of diclofenac sodium (DIC) and papaverine hydrochloride (PAP) after oral administration of composed tablets to rabbits was developed. HPLC method for determination of DIC and PAP in rabbit plasma was developed and validated. Chromatographic separation of DIC, PAP and the IS was achieved on a Zorbax SB C18 5-µm column (150 mm × 4.6 mm) using methanol-water (55 : 45, v/v) as mobile phase at a flow rate of 0.8 mL/min. Pharmacokinetic analysis showed that oral administration of a tablet composed of DIC and PAP do not change the pharmacokinetic parameters such as MRT, MAT, Cl and bioavailability of the active substances compared with single administration of DIC and PAP after single dose.

Keywords: pharmacokinetics, diclofenac sodium, papaverine hydrochloride, rabbits, HPLC

Diclofenac sodium is a nonsteroidal anti-inflammatory drug (NSAID) that it used for the treatment of rheumatic diseases, minor and medium pain, and post surgery analgesia in medicine. Patients who are given formulations of diclofenac or other NSAIDs as a therapeutic strategy often suffer from the gastrointestinal tract complications (1-3). In order to decrease the adverse effects or to increase the therapeutic analgesic effect, diclofenac was orally administered in composed tablets comprising also misoprostol (4) and topically in a formulation comprising e.g., a spasmolytic agent - papaverine hydrochloride (5). In the literature can be found reports of a separate administration of diclofenac and papaverine for the relief of pain in patients with renal colic (6, 7). There are no reports of a formulation comprising diclofenac sodium and papaverine hydrochloride in a composed dosage form, therefore, we decided to prepare tablets consisting of DIC and PAP for oral administration for analgesic effect. Next, the tablets comprising the two drugs were patented (8).

In the literature, there are no reports regarding the relationship of pharmacokinetic parameters between DIC and PAP. Diclofenac is almost completely absorbed after oral administration, it is subjected to first-pass metabolism so that about 50% of the drug reaches the systemic circulation in the unchanged form. More than 99% is bound to plasma proteins, primarily to albumin (3, 9, 10). Diclofenac exhibits a terminal half-life of 1-2 h (3, 9). Metabolism of diclofenac is mediated by both glucuronidation and oxidative biotransformation (11). The oxidative metabolism of diclofenac is catalyzed by two enzymes of the cytochrome P450 family, namely CYP2C9 and CYP3A4 (12). Diclofenac is metabolized to 4'-hydroxydiclofenac and 5-hydroxydiclofenac, 3'-hydroxydiclofenac and 4',5-dihydroxydiclofenac (13, 14). They are then excreted in the form of glucuronide and sulfate conjugates, mainly in the urine (about 60%) but also in the bile (about 35%) and less than 1% is excreted as unchanged diclofenac (3).

Papaverine is completely absorbed by the gastrointestinal tract and metabolized principally by the liver. Papaverine in about 90% bound to plasma proteins. The biological half-life is reported to be between 1 and 2 h (3). The major route of biotransformation is O-demethylation to the corresponding

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phenolic metabolites (15). Several phenolic metabolites have been identified: 4'-desmethylpapaverine (which predominantes in most cell systems), 6-desmethylpapaverine, 7'-desmethylpapaverine, 4',6-didesmethylpapaverine and 3'-desmethylpapaverine (16-18). Papaverine is excreted in the urine, almost entirely as glucuronide-conjugated phenolic metabolites (3).

To test the pharmacokinetics and bioavailability of DIC and PAP given in a single formulation, it was necessary to develop a method for determination of DIC and PAP in rabbit plasma. According to the literature, several HPLC methods using different clean-up procedures, including direct injection, protein precipitation (19-21), solid-phase extraction (SPE) (22), liquid-liquid extraction (LLE) (23-26) and column switching (27) with UV (24-29), fluorimetric (20) or electrochemical detection (30) HPTLC (31) and HPLC-MS/MS (32), GC-MS (33, 34) were applied to determine diclofenac in animal or human plasma. Papaverine hydrochloride in biological fluids was assayed using HPLC (35-37), GLC (38), and GC (39) methods.

There are no data about pharmacokinetics of diclofenac sodium and papaverine hydrochloride after administration in composed formulation and there is no HPLC method for simultaneous determination of diclofenac sodium and papaverine hydrochloride levels in plasma. Therefore, the aim of this study was to examine the pharmacokinetics of DIC and PAP after oral administration of composed tablets to rabbits and develop and validate the HPLC method.

EXPERIMENTAL

Reagents and chemicals

Diclofenac sodium (DIC) was produced by Caesar and Loretz, GmbH, Hilden, Germany, papaverine hydrochloride (PAP) was purchased from Galfarm PPH, Cefarm Lublin, Poland, and phenacetin (IS) from POCh Gliwice, Poland. Polyvinylpyrrolidone K 22 (PVP), mannitol (M) and potato starch (PS) were the products of Merck, Germany. Chloroform was obtained from POCh Gliwice, Poland. Methanol and water (HPLC grade) were purchased from Merck, Germany and other reagents were of analytical grade.

Preparation of tablets

Composition and preparation of tablets (T) containing DIC and PAP were described in the patent (8). Tablets containing only one substance, DIC (T-DIC) or PAP only (T-PAP), were obtained in the same manner as described in this patent, but composition contained only one active substance.

One dose of tablets (T) consists of 50 mg DIC, 20 mg PAP and excipients such as 70 mg PVP, 70 mg M and 90 mg PS to obtain 300 mg of weight. One dose of tablets (T-DIC) consists of 50 mg DIC and excipients to obtain 280 mg of weight. One dose of tablets (T-PAP) consists of 20 mg PAP and excipients to obtain 250 mg of weight.

Tablets were obtained by direct compression of granules, which were previously prepared by wet granulation method. Granules were obtained by mixing and wetting of the powders with PVP solution until a mass of suitable consistency was obtained. Then, the wet mass was granulated using a

Table 1. Physical properties of prepared tablets.

Test	Results		
	T	T-DIC	T-PAP
Weight (mg) mean SD	300.54 ± 2.45	281.32 ± 1.55	249.68 ± 1.87
Thickness (mm) SD	4.02 ± 0.02	3.85 ± 0.01	3.8 ± 0.02
Disintegration time (min) SD	7 ± 2.5	6 ± 1.5	5 ± 2.1
Hardness (kG/mm^2), SD	0.105 ± 0.01	0.1 ± 0.01	0.105 ± 0.03
Friability (%)	0.09	0.22	0.16
Drug content (%) DIC, SD (%) PAP, SD	99.08 ± 1.17 100.05 ± 1.76	100.04 ± 3.93	97.35 ± 1.23

rotary granulator (Erweka, Germany) and a 1.6 mm mesh screen. Granules were dried in a hot air oven (Memmert INB-500) at 40°C for 1 h. The dried granules (moisture 3-5%) were sieved through a 1.6 mm mesh screen. The tablets were obtained from these granules in a press tableting machine (Erweka, Germany) with 9 mm concave punches.

Physical properties of tablets

The tablets were tested as per standard procedure for weight variation (n = 20), thickness (n = 20), hardness (n = 6), friability (n = 20), disintegration time (n = 6) and drug content (n = 10) (Table 1).

Weight uniformity test

For each formulation, twenty tablets were selected randomly and weighed together and their mean weight was calculated. Next, they were individually weighed using a weighing balance (Ohaus AV 513C, USA).

Tablet dimensions

Tablet diameter and thickness were measured using a Vernier Caliper (Digital Caliper 0-150 mm, Comparator).

Hardness test

Hardness of tablet was determined by using an Erweka tablet hardness tester (Erweka, Germany).

Friability test

An Erweka friabilator (Erweka, Germany) was used for the test. Twenty tablets were weighed, subjected to attrition at 25 rpm for 4 min and reweighed afterwards. The percentage loss in weight equivalent to friability was calculated from the equation below: Friability (%) = (loss in weight/initial weight) × 100.

Disintegration time

Disintegration time was measured by using an Erweka apparatus (Germany). Each of six tablets was put into a basket-rack in a vessel and was covered with a disk. After the apparatus had been turned on, the disintegration time of the tablets was observed.

Drug content analysis

Ten tablets from each series were selected at random, weighed together and the mean weight was determined. The tablets were crushed together and weighed exactly 300 mg (T), 280 mg (T-DIC) or 250 mg (T-PAP), when in powder form (n = 6), dissolved in methanol in a 50 mL volumetric flask, filtered using the Whatman filter and appropriately diluted with methanol. Drug content of DIC and PAP were analyzed by HPLC method published in earlier report (40).

Chromatographic conditions

The chromatographic separation of DIC, PAP and the IS (phenacetin) was achieved with a Perkin Elmer 200 HPLC system consisting of the 200 series pump, the 200 series autosampler equipped with a 200 µL loop, the 200 series UV/VIS detector, the 200 series vaccum degasser and a Zorbax SB C18 5-µm column (150 × 4.6 mm, Agilent, USA).

The mobile phase consisted of methanol and water (55 : 45, v/v), and the flow rate was 0.8 mL/min. The injection volume was 100 µL. The UV detection wavelengths at maximum absorbance of DIC and PAP at 280 nm and 238 nm, respectively, were chosen. The samples were injected twice because the Perkin Elmer 200 HPLC system enabled a detection for only one wavelength.

Calibration standards

Stock solutions of DIC (250 µg/mL) and PAP (100 µg/mL) were prepared in mobile phase solution (methanol-water, 55 : 45, v/v) and stored at 4°C wrapped in aluminum foil. The working solutions at concentrations of 0.5, 1.25, 2.5, 5, 12.5, 25 and 50 µg/mL of DIC and 0.5, 1, 2, 5, 10, 20, 40 and 60 µg/mL of PAP were prepared by serial dilution of DIC or PAP stock solutions in mobile phase. The IS working solution (20 µg/mL) was also prepared by dilution of the IS stock solution (100 µg/mL) with mobile phase.

The calibration standards were obtained by appropriate dilution of working solutions with mobile phase. The calibration standards were prepared on the same day at concentrations of 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL of DIC and 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 µg/mL of PAP, by spiking 1 mL blank rabbit plasma with 100 µL of DIC or PAP working solutions and 100 µL of IS working solution.

Sample and calibration standard preparation

Briefly, a 1 mL of blank rabbit plasma, spiked plasma (calibration standard sample) or pharmacokinetic study plasma sample was initially spiked with 100 µL of IS working solution and then 1.5 mL of 1 M hydrochloric acid was added. The mixture was shaken for the period of 10 min. To the obtained mixture, 3 mL of chloroform was added, shaken for 10 min and centrifuged at 3600 rpm for 10 min. The supernatant was separated and evaporated to dryness under nitrogen gas. The residue was reconstituted

with 400 µL of mobile phase and 100 µL aliquot of resulting solution was injected into HPLC apparatus. The samples setting into the vials were immediately assayed. Having obtained a chromatogram at a wavelength of 280 nm, 100 µL of the aliquot was injected once again and assayed at a wavelength of 238 nm for the assay of DIC and PAP, respectively.

Selectivity

The selectivity of the method was tested by comparing the chromatograms of blank plasma controls from six rabbits with that of plasma spiked with DIC, PAP and IS.

Sensitivity

The calibration standard at the lowest concentration, yielding a precision with relative standard deviations (RSD) less than 20% and accuracy within 20% of the nominal concentration, was considered to be the lower limit of quantification.

Calibration curve

Calibration standards at concentrations of 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL of DIC and 0.05, 0.1, 0.2, 0.5, 1, 2, 4 and 6 µg/mL of PAP (n = 2) were freshly prepared as described above and assayed on the same day. This assay was repeated for five consecutive days with freshly prepared calibration standards. The subsequent five calibration curves (y = ax + b), represented by the plots of the peak area of DIC to IS or PAP to IS (y) *versus* the concentration of the calibration standards (x), were generated and obtained by linear least-squares regression as the mathematical model.

Accuracy and precision

In order to validate the intra-day accuracy and precision, the samples of DIC (0.05, 0,25, 1.25 and 5 µg/mL) and PAP (0.05, 0.2, 2, 6 µg/mL) were freshly prepared and analyzed on the same day (n = 5). To validate the inter-day accuracy and precision, the intra-day accuracy and precision assay was repeated for three consecutive days. Data are presented in Table 2.

Accuracy was calculated by comparing the average concentration found to the known concentration, and was expressed in percentage as relative error. Precision was evaluated by calculating the RSD of measured concentrations at each level.

Recovery

The extraction recoveries of DIC and PAP from rabbit plasma at levels (0.05, 0.25, 1.25 and 5 µg/mL of DIC and 0.05, 0.2, 1, 4 µg/mL of PAP) were determined by comparing mean peak area of DIC/IS or PAP/IS samples to that of unextracted DIC or PAP standards in mobile phase at equivalent DIC or PAP level spiked with IS and expressed in percentage, respectively.

Stability of samples

Short-term stability of plasma samples was examined by supplementing blank plasma with appropriate amounts of working solutions of DIC and PAP to obtain 0.05, 0.25, 1.25, 5.0 µg/mL of DIC and 0.05, 0.2, 2.0, 6.0 µg/mL of PAP. Each sample was analyzed at room temperature for 48 h (every 4 h), including their residence time in an autosampler.

Table 2. Intra- and inter-day validation of the method (precision and accuracy) (n = 5).

Intra-day				Inter-day		
Added concentration (µg/mL)	Found concentration (µg/mL)	Precision (% RSD)	Accuracy (relative error, %)	Found concentration (µg/mL)	Precision (% RSD)	Accuracy (relative error, %)
DIC						
0.05	0.0477	7.6	4.6	0.0464	5.9	7.2
0.25	0.2395	3.3	4.2	0.2368	4.0	5.3
1.25	1.1588	4.4	7.3	1.1738	2.1	6.1
5	4.6752	4.5	6.5	4.8122	1.7	3.8
PAP						
0.05	0.0474	8.7	5.2	0.0482	7.6	3.6
0.2	0.1954	3.4	2.3	0.1952	4.5	2.4
2	1.8463	2.3	7.7	1.8823	5.2	5.9
6	5.4871	7.0	8.5	5.6401	4.8	6.0

The *long-term freezer stability* of DIC and PAP in rabbit plasma was assessed by analysis after 30 days of storage at -20°C. The concentration of the stability of the samples (0.05, 0.25, 1.25, 5.0 μg/mL for DIC and 0.05, 0.2, 2.0, 6.0 μg/mL for PAP) was compared to the mean of back-calculated values for the standards at the appropriate concentration from the first day of the long term stability testing.

The long-term freeze-thaw stability of the active substances at concentrations 0.05, 0.25, 1.25, 5.0 μg/mL for DIC and 0.05, 0.2, 2.0, 6.0 μg/mL for PAP in plasma was analyzed in triplicate after 1, 2 and 30 days of storage at -20°C.

Pharmacokinetic study

Animals

The experiments were carried out on randomly selected male Berg rabbits weighing 2.5-3.0 kg, purchased from the licensed breeder (Lisowski, Parczew, Poland). The animals were kept in cages (one per cage) on a 12-hour day/night cycle with free access to food and water. Each experimental group consisted of 9-12 animals. The experimental protocol was approved by the Local Ethics Committee at the Medical University of Lublin (license number 36/2007). All procedures were performed in compliance with the requirements of European convention for the protection of vertebrate animals used for experimental and other scientific purposes (ETS123, 1991).

Administration and sampling

Before the experiment, the rabbits were fasted for the period of 12 h but water was available.

On the first day of the test, the rabbits were divided into four groups and each group consisted of three animals. DIC and PAP used for intravenous administration were separately dissolved in sterile apyrogenic saline. The first group of animals was given a single intravenous injection of sterile apyrogenic physiological saline (as negative control), the second group was administered DIC at a dose of 12.5 mg/kg body weight (b.w.), the third group was injected with PAP at a dose of 5 mg/kg b.w. and the fourth group with DIC (12.5 mg/kg b.w.) and PAP (5 mg/kg b.w.). Intravenous administration was performed in the marginal ear vein of the rabbits at a volume of 0.5 mL/kg b.w. with sterile disposable syringes and needles.

The next day, other rabbits were divided into three groups and each group consisted of three animals. The first group was orally administered a tablet with DIC (12.5 mg/kg b.w.), the second group was given a tablet with PAP (5 mg/kg b.w.) and the third group a tablet composed of DIC (12.5 mg/kg b.w.) and PAP (5 mg/kg b.w.). The tablets were administered *via* an orogastric tube with 100 mL of water. Blood samples were withdrawn from the ear vein at different time intervals: 0, 0.5, 1, 2, 3, and 4 h. These intervals were chosen because the biological half-time of DIC and PAP is 1-2 h (3). In humans, dogs and pigs the terminal elimination half-lives ($t_{1/2â}$) of diclofenac have been reported to range from 1.1 to 2.4 h (9, 41, 42). The blood was collected into heparinized tubes. Plasma was separated and stored at -20°C till the analysis was carried out. All of the plasma samples were analyzed within a week after the separation.

After a lag period of 10 days, the animals were crossed over and the experiments were repeated. Finally, DIC and PAP were administered intravenously and orally in single and composed preparations six times (n = 6). The experiment was carried out at room temperature. All preparations were administered in the morning.

Calculations and statistics

The non-compartmental pharmacokinetic analysis of DIC and PAP concentrations in plasma *versus* time data were calculated using WinNonlin 1.1 software. The plasma concentration – time profiles following a single *i.v.* or *p.o.* doses were not adequately fitted with a compartmental model.

Statistical analysis was carried out using SAS 9.1.3 (SAS Institute, Cary, NC, USA). The data obtained were subjected to statistical analysis using one-way ANOVA and Student's *t*-test and a *p* value of < 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

HPLC method development

Different mobile phases were used to determine either DIC or PAP in biological fluids by HPLC (23-28, 34-36). There is no mobile phase for simultaneous determination of DIC and PAP in biological matrices. In our previous study (40) on simultaneous determination of DIC and PAP in composed tablets, the HPLC method with a mobile phase (methanol-water, 60 : 40, v/v) was developed. Bearing those studies in mind, we tried to achieve a proportion of solvents with an optimized separation of the active substances. It was not easy because the solubility of DIC and PAP is different (3). Various proportions of methanol-water solvents in mobile phase for determination of DIC in human plasma by HPLC method can be found in the literature (30, 43). In our study, methanol-water (55 : 45, v/v) was selected as the mobile phase for determination of

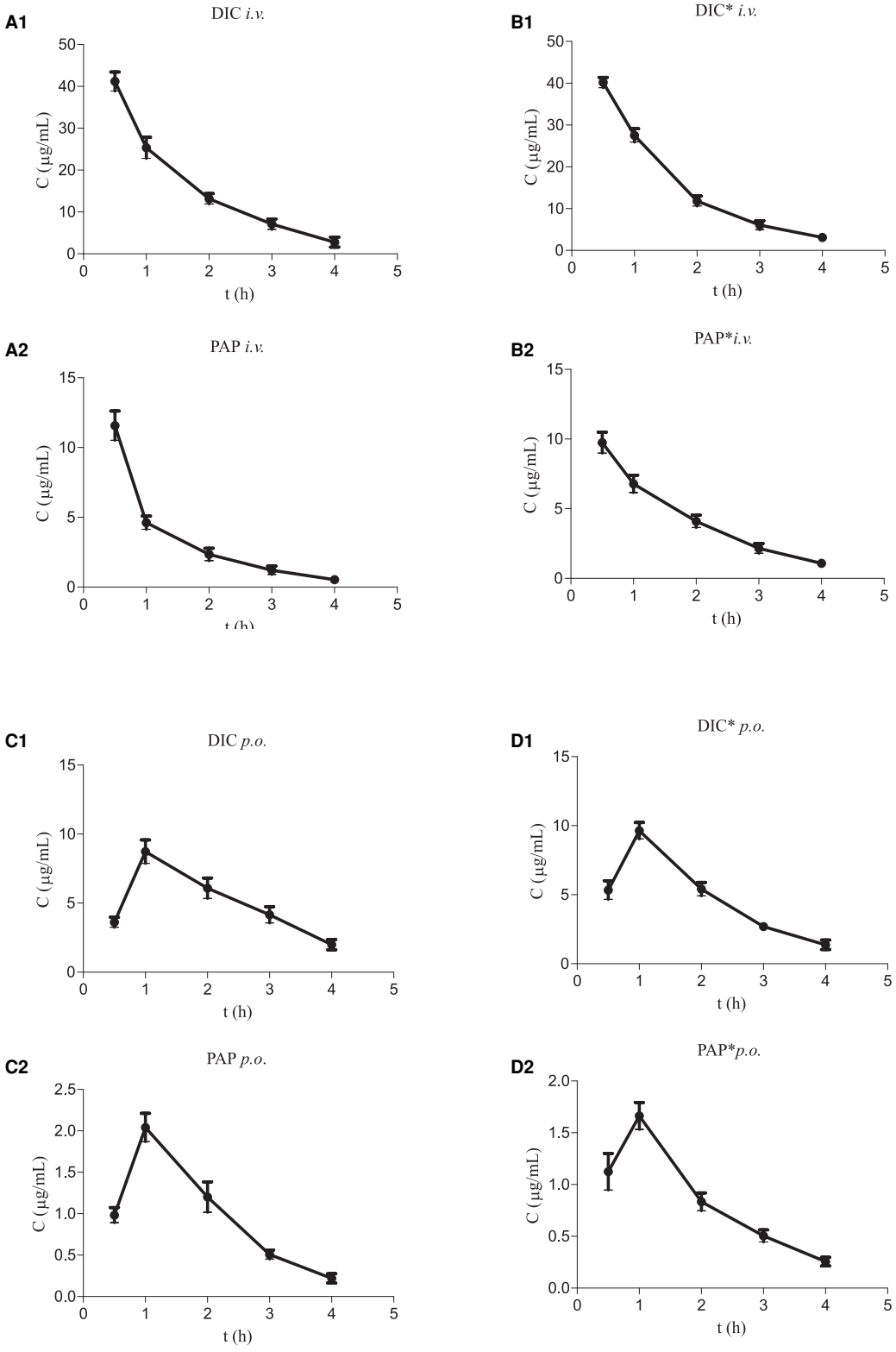

Figure 1. Representative chromatograms of: (A1) DIC (4 µg/mL), PAP (2 µg/mL) and phenacetin (IS) (6 µg/mL) standards at 280 nm and (A2) at 238 nm; (B1) blank rabbit plasma at 280 nm; (B2) blank rabbit plasma at 238 nm; (C1) rabbit plasma sample at 0.5 h after oral administration of composed tablet comprising DIC and PAP and spiked with IS at 280 nm and (C2) at 238 nm; (D1) DIC for LLOQ; (D2) PAP for LLOQ

DIC and PAP in rabbit plasma because of its very simple composition, and clear baseline separation of DIC, PAP and IS was obtained (Fig. 1).

A number of NSAIDs have been used as an internal standard for the analysis of DIC from the biological matrices (19, 21, 31). Several substances were tested as IS. There were very poor baseline separations of ibuprofen, naproxen, ketoprofen or indomethacin from DIC under the experimental conditions used in the present study. However, using phenacetin, clear line separation was achieved. Phenacetin seemed to be the most appropriate drug

Table 3A. Stability of DIC in rabbit plasma samples.

	Short-term stability (48 h) in an autosampler			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5.0
Mean	0.055	0.2402	1.37	5.31
SD	0.0013	0.0029	0.0351	0.0922
CV (%)	2.36	1.21	2.56	1.74
	Long-term freezer stability (30 days)			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5
Mean	0.048	0.2576	1.29	5.15
SD	0.0019	0.0057	0.0521	0.2138
CV (%)	3.96	2.21	4.04	4.15
	Long-term freeze-thaw stability after three cycles (-20°C, 30 days)			
Concentration of DIC (μg/mL)	0.05	0.25	1.25	5
% of the initial conc. of DIC after 30 days	97.43	98.32	97.12	98.89
SD	2.14	1.68	1.89	2.21
CV (%)	0.02	0.02	0.02	0.02

Table 3B. Stability of PAP in rabbit plasma samples.

	Short-term stability (48 h) in an autosampler			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
Mean	0.053	0.2073	2.09	6.25
SD	0.001	0.0034	0.0321	0.0822
CV (%)	1.89	1.64	1.54	1.32
	Long-term freezer stability (30 days)			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
Mean	0.0512	0.2155	2.11	6.17
SD	0.0023	0.0098	0.0821	0.2492
CV (%)	4.49	4.55	3.89	4.04
	Long-term freeze-thaw stability after three cycles (-20°C, 30 days)			
Concentration of PAP (μg/mL)	0.05	0.2	2.0	6.0
% of the initial conc. of PAP after 30 days	98.32	99.03	95.78	98.16
SD	2.5	1.98	2.08	2.41
CV (%)	0.03	0.02	0.02	0.02

Table 4. Pharmacokinetic parameters of DIC and PAP in single or composed formulations administered intravenously or orally.

Parameters	Intravenous				Oral			
	Single		Composed		Single		Composed	
	DIC	PAP	DIC	PAP	DIC	PAP	DIC	PAP
$AUC_{0\rightarrow\infty}$ (h·mg/L) (mean n = 6, ± SD)	83.88 ± 18.78	21.46 ± 5.71	80.34 ± 12.54	22.24 ± 4.65	24.32 ± 5.57	4.12 ± 1.02	21.52 ± 4.36	3.77 ± 0.66
MRT (h) (mean n = 6, ± SD)	1.30 ± 0.45	0.96 ± 0.33	1.32 ± 0.30	1.56 ± 0.38	2.68 ± 0.66	1.82 ± 0.19	2.16 ± 0.71	2.14 ± 0.30
CL (L/h) (mean n = 6, ± SD)	0.49 ± 0.13	0.78 ± 0.23	0.49 ± 0.07	0.74 ± 0.21	0.51 ± 0.09	0.79 ± 0.18	0.49 ± 0.11	0.72 ± 0.20
MAT (h) (mean n = 6, ± SD)					0.72 ± 0.20	0.86 ± 0.43	1.03 ± 0.83	0.76 ± 0.27
F (mean n = 6, ± SD)					0.30 ± 0.07	0.21 ± 0.11	0.28 ± 0.08	0.18 ± 0.07

Pharmacokinetic parameters of DIC and PAP, administered intravenously and orally showed no statistically significant difference between single or composed formulations ($p > 0.05$).

in the present assay and was chosen because it is stable and does not endogenously exist in plasma. Moreover, it did not interfere with the rabbit plasma sample and it was well separated from DIC and PAP.

Liquid-liquid extraction method was used for sample preparation. When it comes to extracting DIC from plasma after the acidification of the sample, hexane-isopropanol at different ratios (44, 45), acetonitrile (19, 46), and dichloromethane (30) were described in the literature. In our study, chloroform was used as solvent for extraction after acidification of plasma sample, because DIC and PAP are soluble in acidic medium and IS in chloroform. This solvent was adopted due to its high extraction efficiency and little interference.

HPLC method validation

Selectivity

No interference of endogenous and extraneous peaks with DIC, PAP and IS at their respective retention times at two wavelengths (RT_{DIC} = 2.87 min, RT_{IS} = 4.77 min and RT_{PAP} = 13.15 min at 280 nm and RT_{PAP} = 13.14 min, RT_{IS} = 4.76 min, RT_{DIC} = 2.85 min at 238 nm) in blank rabbit plasma was observed, as shown in Figure 1.

Sensitivity

The LLOQ of DIC and PAP in 1 mL rabbit plasma was observed to be 0.05 µg/mL.

The limit of detection (LOD) for DIC amounted to 0.015 µg/mL and for PAP to 0.02 µg/mL, based on a signal to noise ratio > 3.

Linearity of calibration curve

The method has a good linearity over the range of 0.05-5 µg/mL for DIC and 0.05-6 µg/mL for PAP. The mean regression equation from five replicate calibration curves was y = 0.1927 (± 0.0415) x + 0.0011 (± 0.00062) for DIC and y = 0.1449 (± 0.0267) x + 0.0033 (± 0.0017) for PAP. The square root of mean correlation coefficient (r^2) was 0.9974 for DIC and 0.9986 for PAP.

Precision and accuracy

The intra- and inter-day precision and accuracy validation with samples of DIC and PAP are presented in Table 2. In the range of 0.05-5 µg/mL DIC, the intra- and inter-day assay precision (RSD) varied from 3.3 % to 7.6% and from 1.7% to 5.9%, respectively. The intra- and inter-day accuracy (relative error) ranged from 4.2% to 6.5% and from 3.8% to 7.2%, respectively.

In the range of 0.05-6 µg/mL PAP, the intra- and inter-day assay precision (RSD) varied from 2.3% to 8.7% and from 4.5% to 7.6%, respectively. The intra- and inter-day accuracy (relative error) ranged from 2.3% to 8.5% and from 2.4% to 6.0%, respectively. The RSD of intra- and inter-day variability was less than 10%, which is within the limit

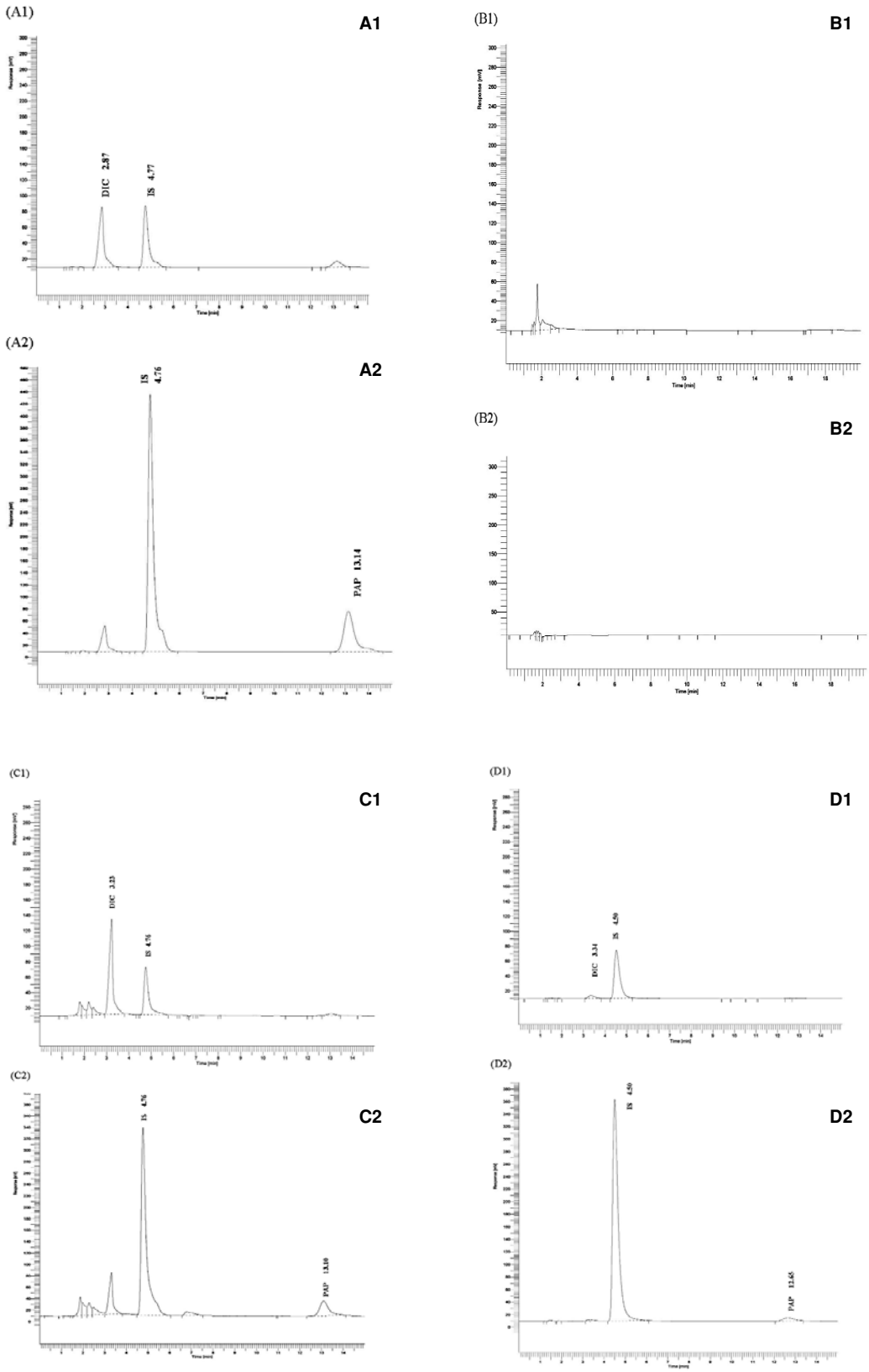

Figure 2. Mean plasma concentration-time profile (n = 6) for DIC and PAP in rabbit plasma after an intravenous (*i.v.*) or oral (*p.o.*) administration of single (DIC, PAP) or composed (DIC*, PAP*) formulations

of acceptability (± 15%) (24). These results indicate that this method had good precision and accuracy.

Recovery

The mean extraction recoveries of DIC were found to be 95.78% ± 2.98 of 0.05-5 µg/mL, PAP 93.09% ± 3.29 of 0.05-6 µg/mL and IS 95.93% ± 1.38 of 5-20 µg/mL, respectively. The recoveries were not dependent on the concentration and consequently resulted in good linearity of the calibration curves.

Evaluation of stability of samples

Stock solutions of DIC (250 µg/mL), PAP (100 µg/mL) and IS (100 µg/mL), and working solutions of DIC, PAP and IS were observed to be over 99% of the nominal concentrations after 5 days of storage at 4°C, compared with freshly prepared solutions. Similarly, working solutions of DIC, PAP and IS were over 99 % of the nominal concentrations after 20 days of storage at -20°C.

Short-term stability of active substances in plasma were investigated at room temperature for 48 h. After the storage, the coefficients of variation (CV, %) values varied from 1.21 to 2.56 for DIC solutions and from 1.32 to 1.89 for PAP concentrations.

Long-term freezer stability of plasma samples (after 30 days of storage at -20°C) CV values for analyzed DIC samples: 0.05, 0.25, 1.25, 5.0 µg/mL amounted to 3.96, 2.21, 4.04 and 4.15%, respectively. PAP (CV) values amounted to 4.49, 4.55, 3.89 and 4.04% for concentrations: 0.05, 0.2, 2.0, 6.0 µg/mL, respectively.

After storage in *long-term freeze-thaw stability* for three cycles (-20°C, 30 days) the drugs were regarded as stable if more than 90% was intact at the end of the study period. The amount of the initial concentration of DIC and PAP remaining after this time was: 97.43 ± 2.14%, 98.32 ± 1.68%, 97.12 ± 1.89%, 98.89 ± 2.21% for DIC concentrations of 0.05, 0.25, 1.25 and 5.0 µg/mL, respectively, and 98.32 ± 2.5%, 99.03 ± 1.98%, 95.78 ± 2.08% and 98.16 ± 2.41% for PAP concentrations of 0.05, 0.2, 2.0 and 6.0 µg/mL respectively.

The results from the stability tests (Tables 3 A and B) indicated that DIC and PAP were stable under the conditions studied.

Pharmacokinetic study

The mean plasma concentration-time profiles of DIC and PAP are shown in Figure 2. The pharmacokinetic parameters of DIC and PAP were calculated by non-compartmental analysis and are presented in Table 4. More than 99% of diclofenac is bound to plasma proteins (3, 9) and papaverine is about 90% (3), therefore, we expected changes in the pharmacokinetic parameters of substances after their co-administration. As compared with the pharmacokinetic parameters, such as $AUC_{0\to\infty}$, MRT and CL of DIC or PAP administered intravenously and orally, after single dose there was no statistically significant difference between single or compound formulations ($p > 0.05$). The MAT of DIC and PAP administered as single or compound formulations were calculated to be over 1 h and these MAT values presented no statistically significant difference between the formulations ($p > 0.05$). Similarly, after the oral administration of DIC to the rabbits, the mean MAT was equal to 1.24 h (47). Diclofenac penetrates to synovial fluid where concentrations may persist even when in plasma concentrations fall. Diclofenac is metabolized by cytochrome P450 by phase I hydroxylation and by phase II conjugation with glucuronic acid and the amino acid taurine. Diclofenac is extensively metabolized in camels as well as in humans, goats and cattle, and this appears to be mediated by cytochrome P450 2C subfamily (48, 49).

Drug-protein binding has an influence on the distribution equilibrium of drugs. Only the free, non-protein bound fraction of drug can leave the circulatory system and diffuse into tissue. The equilibrium between free drug can be maintained over a relatively long period of time because of the dissociation of the drug-protein complex. The transport function of plasma proteins is of importance for drugs of low solubility in water. Plasma protein binding is of significant influence on the distribution equilibrium if the drug is polar and therefore diffuses slowly into tissue. If, in addition, such a substance has a high affinity for plasma proteins, displacement from its protein binding sites may result in a change of distribution equilibrium and an altered pharmacologic response (50).

The $AUC_{0\to\infty}$ values of DIC or PAP after intravenous injection were higher than after oral administration. It was shown that bioavailability of (F) DIC and PAP after oral administration was decreased to over 30% for DIC and 20% for PAP, but for some rabbits bioavailability amounted to over 43% for both DIC and PAP. When DIC is orally administered, it is almost completely absorbed due to the fact that it is subject to first-pass metabolism. In this way about 50% of the drug reaches the systemic circulation in the unchanged form (3).

The metabolic tracts of both of the substances showed that it is possible that a simultaneous admin-

istration of DIC and PAP has no influence on their pharmacokinetics.

In our study, oral administration of a tablet comprising DIC and PAP did not change the main pharmacokinetic parameters of the active substances after single dose. Similarly, administration of diclofenac and misoprostol in one composed tablet did not change the pharmacokinetics of these two drugs (4).

CONCLUSION

Tablets comprising DIC and PAP were prepared and all of tablets fulfilled pharmacopoeal requirements such as average weight, hardness and assay of drug content. A HPLC-UV method with LLE extraction was developed and validated for the quantitative determination of DIC and PAP in rabbit plasma. The LLOQ of the method was 0.05 μg/mL and the sensitivity was compared with previously reported HPLC-UV methods using LLE (21) and protein precipitation (19). The proposed method was successfully applied to determination of DIC and PAP, after injection and oral administration to rabbits, for use in pharmacokinetic study. A non-compartmental pharmacokinetic analysis showed that oral administration of composed tablet comprising DIC and PAP did not change the main pharmacokinetic parameters such as MRT, MAT, CL and bioavailability of active substances compared with a single administration of DIC and PAP after single dose.

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EVALUATION OF SORPTIVE PROPERTIES OF VARIOUS CARRIERS AND COATING MATERIALS FOR LIQUISOLID SYSTEMS

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Abstract: The basic principle of liquisolid systems formulation lies in the conversion of the drug in a liquid state into an apparently dry, free-flowing and readily compressible powder by its blending (or spraying) with specific carriers and coating materials. The selection of the most suitable carrier and coating material depends especially on their values of flowable liquid retention potential (Φ), which is defined as the maximum mass of liquid that can be retained per unit mass of powder material, while maintaining an acceptable flowability. The presented work focused on the determination of the maximum amount of propylene glycol (PG), which can be retained by several selected carriers and coating materials while maintaining acceptable flow properties of the liquisolid powder blend. Granulated forms of magnesium aluminometasilicates (Neusilin® US2 and Neusilin® NS2N), dibasic calcium phosphate (Fujicalin®) and microcrystalline cellulose (Avicel® PH 101) were tested due to their frequent use. Powdered forms of magnesium aluminometasilicate (Neusilin® UFL2) and colloidal silica (Aerosil® 200) were used as common coating materials. From the evaluation of liquisolid mixtures with different amounts of liquid, it could be observed that 1 g of Neusilin® US2, Neusilin® UFL2, Neusilin® NS2N, Aerosil® 200, Fujicalin® and Avicel® PH 101 can retain 1.00, 0.97, 0.54, 0.04, 0.25 and 0.12 g of propylene glycol, respectively, while maintaining acceptable flowing properties for further processing.

Keywords: liquisolid systems, flowable liquid retention potential, carrier, coating material, aluminometasilicates, microcrystalline cellulose, Aerosil®, Neusilin®, Fujicalin®

Liquisolid systems (LSS) formulation represents a new, innovative and promising method in the production of solid dosage forms with enhanced *in vitro* dissolution rate and improved *in vivo* bioavailability of poorly soluble drugs. The basic principle of LSS formulation lies in sorption of a drug in liquid state onto specific carriers and coating materials, resulting in formation of apparently dry, free-flowing and readily compressible powder (1). The active ingredients are in liquisolid systems in a similar state as in soft gelatin capsules filled by a liquid drug (non-volatile liquid vehicle is used to dissolve the solid drug and the final preparation does not involve drying or evaporation process) (2). It is well established that these formulations show higher and more consistent bioavailability than conventional oral dosage forms because the active ingredient is already dissolved.

Properties of carriers, such as specific surface area (SSA) and liquid absorption capacity, are the most important factors in the formulation of liquisolid systems because they allow incorporation of a greater amount of the drug in liquid state (3, 4). Therefore, the selection of a carrier mainly depends on its specific surface area, liquid adsorption capacity, flowability and compressibility (5).

In the past, *colloidal silica* (SSA – 200 m^2/g) (6) was used as a carrier material to prepare the predecessors of liquisolid systems called "Powdered Solutions". However, these preparations did not have suitable properties facilitating their compression into tablets (5). Compression enhancers such as microcrystalline cellulose were added to powdered solutions to increase the compressibility of the formulation. Nevertheless, product properties were never standardized to comply with industrial requirements (7). In modern liquisolid system formulations, colloidal silica is used as a coating material absorbing the excessive liquid from the carrier and thus ensuring good flowability of the created admixture (8).

To date, the most commonly used carrier in LSS has been the microcrystalline cellulose (MCC) with SSA about 1.18 m^2/g (9). It has been used to

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prepare liquisolid tablets containing bromhexine hydrochloride (10), ezetimibe (11), flutamide (12), nifedipine (13), valsartan (14), etc. A frequent use of MCC in liquisolid system formulation is caused by its long-term utilization in pharmaceutical industry, low price, good stability and availability in different particle sizes and moisture grades (15). Moreover, in previous studies it was shown that carriers other than MCC, e.g., lactose (SSA – 0.35 m^2/g) (9), sorbitol (SSA – 0.37 m^2/g) (9) and starch (SSA – 0.60 m^2/g) (9), were required in higher amounts for the conversion of a drug in liquid state into dry, non-adherent and free-flowing powdered form (16). This was attributed to a higher specific surface area of MCC (16). However, on the market, there are available several substances with extremely large SSA and significantly higher liquid absorption capacity, such as magnesium aluminometasilicates, anhydrous dibasic calcium phosphate, mesoporous silicates, etc. (17, 18).

The first of them Fujicalin® is a synthetic spherically agglomerated anhydrous dibasic calcium phosphate. The unique production process of Fujicalin® consists of a restricted crystal growth of anhydrous dibasic calcium phosphate followed by spray drying of aqueous dispersion of these microcrystals. Thereby, porous spherical particles containing microcrystals of anhydrous dicalcium phosphate are obtained (19). Its high specific surface area (40 m^2/g), porosity and ability to adsorb up to 1.2 mL/g (17) of liquid while remaining the dry, free flowing and compressible powder makes Fujicalin® a suitable carrier for liquisolid system preparations. This fact was proved by Hentzschel et al. (3), who used Fujicalin® to prepare LSS containing tocopherol acetate as a model drug.

Neusilin®, an amorphous form of magnesium aluminometasilicate, is commercially available in 11 various grades, which differ in their forms (powder, granules), bulk densities, water content, particle sizes and pHs (neutral, alkaline) (20). The application of synthetic magnesium aluminometasilicates has been an objective of several studies. Their absorption properties were used in tobacco industry, for example, where they were added to cigarette filters as aldehyde sorbents (especially formaldehyde) (21). In pharmaceutical technology, they have been used as carriers for solid dispersions to improve the drug dissolution or to granulate oily formulations and to increase the formulation stability (22). Based on its use as an excipient in various formulations, up to 1.05 g Neusilin® can be used in oral formulations per day (23). Moreover, alkaline grades of Neusilin® are approved as antacid active ingredients with a maximum dosage of 4 g/day (23). In liquisolid systems, Neusilins® can be used as carriers (granulated form) and also as coating materials (powdered form). Neusilins® are able to retain a greater amount of a drug in its liquid state than MCC. Disintegration of tablets containing Neusilin® as a carrier is slower because of its poor disintegration properties (24). Recently, Neusilin® US2 represents the most commonly used carrier for LSS formulations from the group of magnesium aluminometasilicates (3, 24). It is prepared by spray drying and thus provides a high porosity, very large SSA (up to 300 m^2/g), high liquid adsorption capacity (up to 3.4 mL/g), and anticaking and flow enhancing properties (20, 25).

Mesoporous silicates were originally developed to be applied as molecular sieves (26). In pharmaceutical technology, they were initially used for the controlled drug delivery (27-29). According to their extremely large specific surface area (up to 1500 m^2/g), large pore size and pore volume (30), ordered mesoporous silicates show a high potential in the preparation of liquisolid systems. Chen et al. (31) used hollow mesoporous silicas (HMS) as carriers for an insoluble drug carbamazepine dissolved in PEG 400. A significant improvement of the drug loading and dissolution rate demonstrated that HMS could form good reservoirs for drug solutions to enhance the dissolution of poorly water-soluble drugs (31).

This work is focused on the determination of capability of several selected carriers and coating materials to absorb propylene glycol by determining their flowable liquid retention potential for this solvent. A granulated form of magnesium aluminometasilicates (Neusilin® US2, Neusilin® NS2N), dibasic calcium phosphate (Fujicalin®) and microcrystalline cellulose (Avicel® PH 101) were used as carrier materials. A powdered form of magnesium aluminometasilicate (Neusilin® UFL2) and colloidal silica (Aerosil® 200) were used as coating materials. Different amounts of propylene glycol were added to carriers/coating materials and flow properties of liquisolid mixtures were evaluated. The flowable liquid retention potential was calculated as a liquid/solid mass ratio of the blend with angle of slide corresponding to 33˚ (4, 32).

MATERIALS AND METHODS

Materials

Magnesium aluminometasilicates (Neusilin® US2, Neusilin® NS2N and Neusilin® UFL2) and anhydrous dibasic calcium phosphate (Fujicalin®) were kindly gifted by Fuji Chemical Industry Co.,

Ltd (Japan). Avicel® PH 101 (FMC Biopolymer, Ireland) and Aerosil® 200 (Eurošarm spol. s.r.o., Czech Republic) were selected as the most commonly used carrier and coating materials. Propylene glycol (Dr. Kulich Pharma, Czech Republic) was used as non-volatile biocompatible solvent with good dissolving properties for many drugs.

Methods

Preparation of liquisolid powders

Liquisolid powders were prepared by a simple blending of carriers/coating materials with propylene glycol. The initial amount of carrier/coating material was selected based on scientific literature according to its characteristics such as specific surface area and adsorption capacity (Table 1). The carrier/coating material was mixed with the liquid vehicle using a mortar and pestle, sieved through the sieve with 1 mm mesh size and subsequently homogenized in a three-axial homogenizer (T2C, TURBULA System Schatr, Switzerland) for 10 min.

The next experimental step was adding of a liquid (usually in amount of 1 g; 0.5 g in the case of Aerosil® 200). The first two addings of PG to Neusilin® UFL2 were 2.5 g, because of the high value of angle of slide (more than 40°). After the evaluation of flow properties and angle of slide, a further amount of a liquid vehicle was added. The whole blend was sieved and homogenized again after each adding of PG. When the value of angle of slide approached 33° (considered as an optimum) (4, 32), at least two more addings of 1 g (0.5 g in case of Aerosil® 200) of propylene glycol were performed.

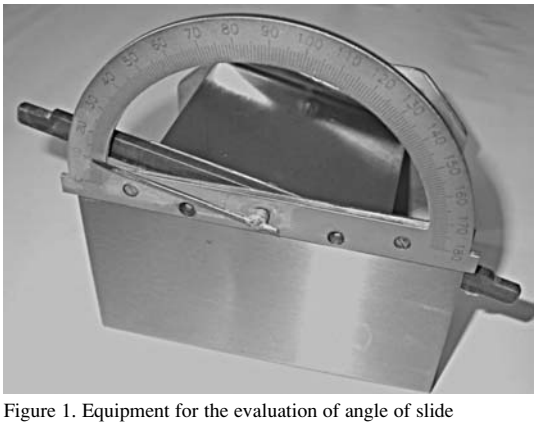

Figure 1. Equipment for the evaluation of angle of slide

Evaluation of angle of slide

Angle of slide is a specific parameter for evaluation of flow behavior of liquisolid mixtures. Spireas et al. (33) suggested that angle of slide is a preferred method to evaluate the flowability of powders with particles smaller than 150 μm.

Angle of slide was used to evaluate the flow properties of carriers/coating materials and liquisolid mixtures. The tested sample (10 g) was placed on one end of a stainless steel plate with a polished surface (Fig. 1). This end was gradually raised until the plate with the horizontal surface formed an angle at which the sample was about to slide. Three determinations were carried out; average and standard deviation were calculated. Angle of slide corresponding to 33° is regarded as optimal (32, 4).

Evaluation of angle of repose

A fixed funnel and a free-standing cone were used to measure the angle of repose. To the funnel (105 mm in diameter, 190 mm high with 105 mm long stem and internal diameter of 5 mm) was introduced 50 g (25 g in case of Aerosil® 200) of the powder mixture. Height (h) and diameter (d) of the cone of powder, which formed after the mixture flew through the funnel, were evaluated and angle of repose (α) was calculated (34). The evaluation was repeated 3 times; average and standard deviation were calculated.

Table 1. Characterizations of carriers/coating materials and their amounts used to prepare liquisolid powders.

Carrier	SSA [m^2/g]	Adsorption capacity [mL/g]	Used amount [g]	Initial amount of PG [g]
Neusilin® US2	300.0 (18)	3.4 (18)	25.0	25.0
Neusilin® UFL2	300.0 (18)	3.4 (18)	30.0	20.0
Neusilin® NS2N	250.0 (18)	2.4 (18)	35.0	15.0
Aerosil® 200	200.0 (6)	2.0 (20)	24.0	1.0
Fujicalin®	40.0 (17)	1.2 (17)	40.0	10.0
Avicel® PH 101	1.1 (9)	1.0 (20)	49.0	1.0

Evaluation of flowability (flow through the orifice)

The total of 50 g (25 g in the case of Aerosil® 200) of the tested powder sample without compacting was introduced into a dry stainless steel funnel with a closed bottom opening (diameter 25 mm) (MEDIPO, Czech Republic according to Eur. Ph.). The bottom opening of the funnel was unblocked and the time the entire sample needed to flow out of the funnel was measured. Three measurements were carried out; average and standard deviation were calculated.

Tapped and bulk density measurements

A required amount of the liquisolid powder was introduced into a dry calibrated cylinder without compacting. The powder was carefully leveled and unsettled apparent volume V_0 was recorded. Bulk density was calculated in compliance with Ph. Eur. 8 (34).

The cylinder was fixed to the base (SVM 102, ERWEKA GmbH, Germany). After 10, 500 and 1250 taps the corresponding volumes V_{10}, V_{500} and V_{1250} were marked. Tapped volumes were used to calculate the tapped density, compressibility index (CI) and Hausner ratio (HR) (34).

Determination of the flowable liquid retention potential (Φ-value)

Carrier/coating material (amount shown in Tab. 1) was mixed with varying amount of propylene glycol, sieved through the sieve with mesh size 1 mm and homogenized in three-axial homogenizer for 10 min. Thereafter, flow properties of these liquisolid mixtures were evaluated. The liquid/solid mass ratio (m/m) of blends with angle of slide corresponding to 33° was regarded as the Φ-value (Equation 1) of the excipient.

$$\Phi = \frac{m_{max}}{Q} \qquad (1)$$

where m_{max} is the maximum amount of liquid that can be retained per unit mass of the powder material (Q) while maintaining acceptable flowability.

RESULTS AND DISCUSSION

Experimental part of the presented work aimed at the determination of the maximum amount of propylene glycol (PG), which can be retained by several selected carriers and coating materials while maintaining acceptable flow properties of the liquisolid powder blend. Granulated form of magnesium aluminometasilicates (Neusilin® US2 and Neusilin® NS2N), dibasic calcium phosphate (Fujicalin®) and microcrystalline cellulose (Avicel® PH 101) were used as carrier materials. A powdered form of magnesium aluminometasilicate (Neusilin® UFL2) and colloidal silica (Aerosil® 200) were evaluated as coating materials. According to the scientific literature dealing with liquisolid systems (2, 35), angle of slide was selected as the main parameter determining flow properties of liquisolid powders. Liquisolid blends with angle of slide close to 33° were used to calculate the liquid retention potential (Φ-value, equation 1). In the case when more similar values of angle of slide close to 33° were obtained for different mixtures with various amounts of propylene glycol, other parameters, such as angle of repose, flowability, compressibility index (CI) and Hausner ratio (HR) were taken under consideration as auxiliary evaluation parameters.

Evaluation of blank carriers and coating materials

The evaluation of Neusilin® US2 flow properties (Table 2) implied that experimentally measured values of angle of slide (35.67 ± 0.58°) were higher than the required angle of 33° (32, 36). The values of angle of repose of 29.66 ± 0.92° were similar to the value presented by the manufacturer (30.00°)

Table 2. Flow properties of carriers/coating materials.

Carrier/coating material	Angle of slide [°]	SD	Angle of repose [°]	SD	Flowability [s/50 g]	SD	CI [%]	HR
Neusilin® US2	35.67	0.58	29.66	0.92	6.21	0.45	10.59	1.11
Neusilin® NFL2	39.33	0.58	41.47	0.67	75.16	3.18	24.75	1.33
Neusilin® NS2N	37.17	0.76	25.87	0.49	7.30	2.19	13.27	1.15
Aerosil® 200	47.00	2.00	NA	NA	8	NA	25.00	1.33
Fujicalin®	23.33	0.76	25.77	0.46	1.16	0.04	6.67	1.07
Avicel® PH 101	48.43	1.53	36.25	0.39	**8**	NA	24.21	1.32

NA = not available.

(20) and indicated excellent flow properties. Other parameters, e.g., compressibility index (10.59%), Hausner ratio (1.11) and flowability (6.21 ± 0.45 s/50 g) referred to its acceptable flow character (34). From Table 2 it is clear that Neusilin® UFL2 showed, due to its powder form, worse characteristics of flow in comparison to granulated forms of aluminometasilicates (Neusilin® US2 and Neusilin® NS2N). This phenomenon is well established also for other excipients (e.g., lactose, calcium sulfate, mannitol, etc.) (37, 38). Blank Neusilin® UFL2 (Neusilin® UFL2 without propylene glycol) also showed higher values of angle of slide (39.33 ± 0.58°) than optimal 33°. Experimentally measured values of angle of repose (41.47 ± 0.67°) were lower than the angle presented by the manufacturer (45.00°) (18) and values of flowability (75.16 ± 3.18 s/50 g), CI (24.75%) and HR (1.33) indicated passable flow properties of this material (34).

Neusilin® NS2N also showed higher values of angle of slide (37.17 ± 0.76°) in comparison to the recommended value of 33°. However other parameters (Table 2), such as angle of repose (25.87 ± 0.49°), flowability (7.30 ± 2.19 s/50 g), CI (13.27%) and HR (1.15) implied its excellent/good character of flow (34).

It was not possible to measure angle of repose and flowability of Aerosil® 200 because of its adhesion to the surface of funnels and cohesion of porous lightweight particles. CI = 25.00% and HR = 1.33 indicated passable flow properties of Aerosil® 200 (34). Angle of slide was 47.00 ± 2.00°, i.e., the second highest value obtained among the tested materials (Table 2).

Evaluation of flow properties of Fujicalin® implied its excellent character of flow (Table 2). Experimentally measured values of angle of repose (25.77 ± 0.46°) and compressibility index (6.67%) were lower than values presented by the manufacturer (29.50° and 15.10%) (17). Fujicalin®, due to its agglomerated character (spray drying preparation), also showed excellent flowability (1.16 ± 0.04) and low value of angle of slide (23.33 ± 0.76°).

Among the selected carriers/coating materials, Avicel® PH 101 showed the highest value of angle of slide (48.43 ± 1.53°). Experimentally measured values of angle of repose (36.25 ± 0.39°) were similar to the value given by Nada et al. (38.00) (39). Flowability of this type of MCC could not be measured and values of CI (24.21%) and HR (1.32) corresponded to passable character of flow (34).

Mixture of Neusilin® US2 with propylene glycol

From Figure 2 it could be observed that the increasing amount of propylene glycol decreased angle of slide of Neusilin® US2/PG mixtures. The blend containing 25 g of PG had the value of angle of slide closest to 33° (32.83 ± 0.29°). Other parameters of this liquisolid blend, such as angle of repose (24.63 ± 2.14°), flowability (2.57 ± 0.26 s/50 g), CI (10.20%) and HR (1.11) also complied with requirements for powders with excellent flow properties (Table 3).

From the evaluation of flow properties it is obvious that the increasing amount of propylene glycol deteriorated angle of repose and flowability of liquisolid blend (Table 3). The results implied that adding of liquid initially decreased values of angle of repose from 24.63 ± 0.00° (25 g PG) to 22.50 ± 0.00 (26 g PG). However, subsequent adding of propylene glycol increased its values up to 29.70 ± 1.78° (blend with 29 g of PG).

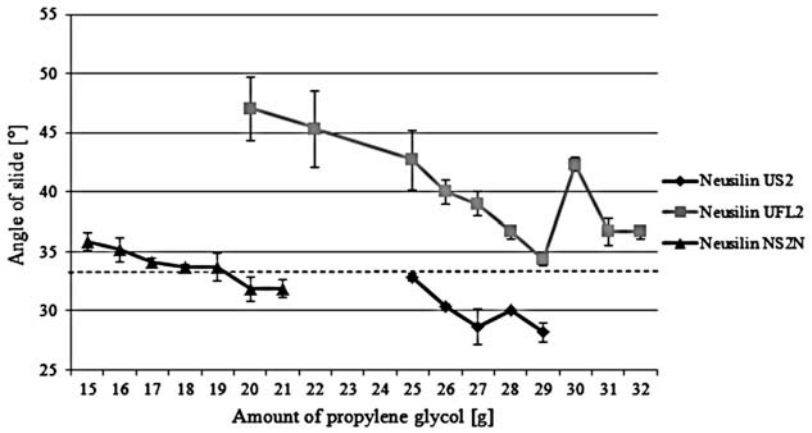

Figure 2. Angle of slide of Neusilins® /PG mixtures. The dashed line indicates the desired angle of slide 33°

Table 3. Flow properties of Neusilin® US2 and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
25	**100**	**24.63**	**2.14**	**2.57**	**0.26**	**10.20**	**1.11**
26	104	22.50	0.00	2.86	0.27	9.38	1.10
27	108	24.47	0.46	3.43	0.54	12.50	1.14
28	112	26.85	0.13	3.30	0.26	13.83	1.16
29	116	29.70	1.78	3.86	0.04	14.13	1.16

* Sample with most suitable properties is written **in bold**. ** In relation to the carrier material.

Table 4. Flow properties of Neusilin® UFL2 and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
20.0	66.7	41.17	0.45	8	NA	23.76	1.31
22.5	75.0	42.70	0.98	8	NA	27.55	1.38
25.0	83.3	43.53	0.29	8	NA	27.08	1.37
26.0	86.7	44.47	0.29	8	NA	26.04	1.35
27.0	90.0	42.03	0.29	8	NA	25.53	1.34
28.0	93.3	36.77	0.64	8	NA	22.89	1.30
29.0	**96.7**	**42.70**	**0.50**	8	**NA**	**25.56**	**1.34**
30.0	100.0	38.67	0.65	8	NA	19.10	1.24
31.0	103.3	34.80	0.70	8	NA	20.88	1.26
32.0	106.7	38.67	0.65	8	NA	22.45	1.29

* Sample with most suitable properties is written **in bold**. ** In relation to the coating material. NA = not available.

The flowability of liquisolid mixture of Neusilin® US2 and PG (maximum value of 3.86 ± 0.04 s/50 g in sample with 29 g of sorbed PG) was enhanced in comparison to flowability of blank Neusilin® US2 (6.21 ± 0.45 s/50 g). As can be observed from Table 3, values of flowability increased from 2.57 ± 0.26 s/50 g (mixture with 25 g of PG) to 3.86 ± 0.04 s/50 g (mixture containing 29 g of PG). This deterioration of powder flow properties could be explained by the oversaturation of carrier with higher amounts of sorbed liquid vehicle.

Compressibility index and Hausner ratio (Table 3) implied the same tendency as angle of repose (Table 3). Initially the values decreased but after adding more PG they increased again. The values correspond to powders with good/excellent flow properties (34).

The flowable liquid retention potential (Φ-value) of Neusilin® US2 mixed with PG was calculated (Equation 1) as the ratio of amount of liquid vehicle (25 g) and weight of carrier (25 g) and is equal to 1.00 (Fig. 3). This experimentally measured Φ-value is four times higher than that given by Shah et al. (40). In their study, angle of repose was used as the main parameter for the evaluation of powder flow properties, which could have caused the significant differences between values of flowable liquid retention potential (40).

Mixture of Neusilin® UFL2 with propylene glycol

Adding of 20 g of PG to Neusilin® UFL2 increased the angle of slide from 39.33 ± 0.58° to 47.00 ± 2.65°. The subsequent adding of propylene glycol decreased the mixture values to 34.33 ± 0.58° (29 g of PG), as shown in Figure 2. However, further additions of liquid vehicle increased the values of angle of slide again. Mixture containing 29 g of PG showed angle of slide 34.33 ± 0.58°, which was the closest value to 33°, regarded as optimum. Other parameters of this liquisolid powder sample, such as angle of repose (47.70 ± 0.50°), CI (25.56) and HR (1.34) referred to passable or poor character of flow (34) (Table 4).

Based on the evaluation of angle of repose, compressibility index and Hausner ratio (Table 4),

there was observed no obvious dependence on the amount of propylene glycol added to Neusilin® UFL2. All the measured values were in the range given by Ph. Eur. 8.0 for fair, passable or poor powder flow properties. The flowability of PG/Neusilin® UFL2 mixtures could not be evaluated. The insufficient character of flow is caused by the presence of Neusilin® UFL2 in its powdered form (18).

The Φ-value for the Neusilin® UFL2 mixed with PG was calculated as the ratio between the amount of liquid vehicle (29 g) and weight of carrier (30 g) and was equal to 0.97 (Fig. 3).

Mixture of Neusilin® NS2N with propylene glycol

From Figure 2 it is clear that the angle of slide decreased with the increasing amount of propylene glycol in PG/Neusilin® NS2N mixtures. The mixture containing 19 g of PG showed the value of angle of slide (33.67 ± 1.16°) closest to 33°. The mixtures' angle of repose (35.99 ± 2.05°) referred to fair flow properties and values of CI (21.28%) and HR (1.27) implied passable character of powder flow (34).

Evaluation of flow properties implied that the increasing amount of propylene glycol deteriorated angle of repose, flowability, CI and HR of liquisolid blend (Table 5).

The flowable liquid retention potential was calculated for the mixture containing 19 g of propylene glycol. The Φ-value for Neusilin NS2N is equal to 0.54 (Fig. 3).

Mixture of Aerosil® 200 with propylene glycol

Figure 4 and Table 6 imply that mixing of Aerosil 200 with propylene glycol improved flow properties of liquisolid mixtures. Values of angle of slide decreased after adding 2 g of PG to 29.00 ±

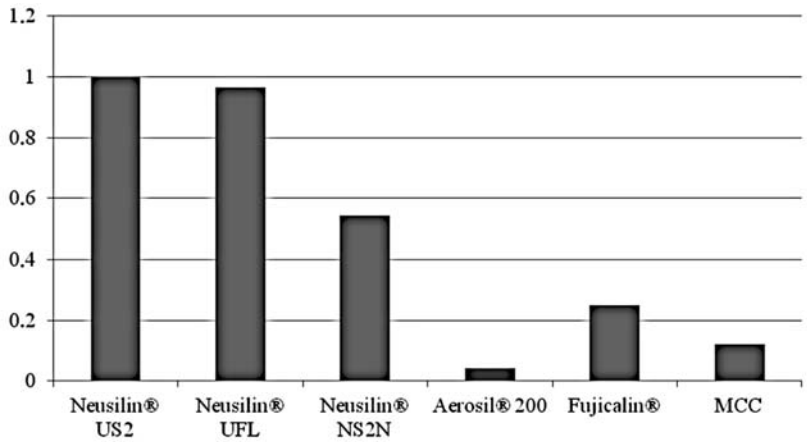

Figure 3. Flowable liquid retention potential (Φ-value) of carriers/coating materials

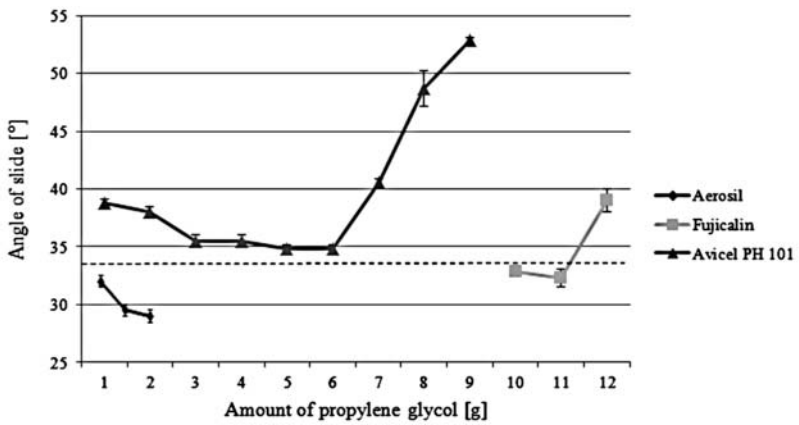

Figure 4. Angle of slide of Aerosil® 200, Fujicalin® and Avicel® PH 101 mixtures with PG. The dashed line indicates the desired angle of slide 33°

0.50°. Mixture containing 1 g of PG revealed the closest value (32.00 ± 0.50°) to the optimal angle of slide of 33°. Other parameters, such as CI (15.29%) and HR (1.18) referred to powders with good character of flow (34).

Angle of repose could not be evaluated. Flowability (Table 6) of liquisolid blends was improved by adding of propylene glycol. Polyethylene glycol could act as a lubricant (41) and hence enhance flowability of powder samples.

Values of CI and HR (Table 6) increased with the increasing amount of PG. Additions of PG caused deterioration of its flow properties from good to fair character of flow.

The Φ-value for the Aerosil® 200 mixed with PG was calculated as the ratio of amount of liquid vehicle (1 g) and weight of carrier (24 g) and is equal to 0.042 (Fig. 3). This experimentally measured value is significantly lower than values described in literature (1.5 (42) and 3.31 (43)). This difference could have been caused by poor homogeneity of the liquisolid blend prepared by simple blending. Higher homogeneity of mixtures could be reached by spraying propylene glycol onto carrier/coating material in the fluid bed equipment.

Mixture of Fujicalin® with propylene glycol

Evaluation of angle of slide (Fig. 4) implied that adding of propylene glycol increased its values in comparison to angle of slide of blank Fujicalin®. Mixture containing 10 g of PG had the closest value of angle of slide (32.83 ± 0.29°) to the recommended 33°. The angle of repose (29.70 ± 2.97°), flowability (1.03 ± 0.09 s/50 g), CI (8.24%) and HR (1.09) of this liquisolid powder sample also met the requirements for powders with excellent flow properties.

Adding of propylene glycol also had a negative effect on values of angle of repose (Table 7). The measured values increased up to 32.60 ± 1.39° (mixture containing 12 g of PG). The lowest angle of repose (27.30 ± 1.21°) had the liquisolid blend with 11 g of PG.

Evaluation of flowability (Table 7) implied that mixing of Fujicalin® with a liquid vehicle decreased its values. The flowability enhancing could be caused by filling of irregularities on carriers' surface with propylene glycol (44).

Values of CI and HR (Table 7) increased after adding PG. However, all values were in the range given by Ph. Eur. 8.0 for excellent character of powder flow.

Table 5. Flow properties Neusilin® NS2N and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
15	42.9	28.61	0.81	9.31	0.55	16.49	1.20
16	45.7	31.97	0.87	10.65	0.69	16.84	1.20
17	48.6	33.91	1.44	12.34	0.31	20.00	1.25
18	51.4	36.38	3.40	11.40	0.44	20.21	1.25
19	**54.3**	**35.99**	**2.05**	**10.37**	**0.24**	**21.28**	**1.27**
20	57.1	35.34	070	9.48	0.27	22.58	1.29
21	60.0	40.11	0.71	8.12	0.96	21.11	1.27

* Sample with most suitable properties is written **in bold**. ** In relation to the carrier material.

Table 6. Flow properties of Aerosil® 200 and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
1.0	**4,2**	**NA**	**NA**	**45.31**	**0.81**	**15.29**	**1.18**
1.5	6,3	NA	NA	24.81	3.31	16.25	1.19
2.0	8,3	NA	NA	24.45	2.63	17.95	1.22

* Sample with most suitable properties is written **in bold**. ** In relation to the coating material. NA = not available.

Table 7. Flow properties of Fujicalin® and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
10	**25.0**	**29.70**	**2.97**	**1.03**	**0.09**	**8.24**	**1.09**
11	27.5	27.30	1.21	0.95	0.05	10.23	1.11
12	30.0	32.60	1.39	1.10	0.07	10.59	1.12

* Sample with most suitable properties is written **in bold**. ** In relation to the coating material.

Table 8. Flow properties of Avicel® PH 101 and PG mixtures.

Amount of PG		Angle of repose	SD	Flowability	SD	CI	HR
[g]	[%]**	[°]		[s/50 g]		[%]	
1	2	35.53	1.68	7.46	0.41	21.96	1.28
2	4	37.83	0.96	5.64	3.26	18.67	1.23
3	6	35.45	0.92	3.07	0.12	18.18	1.22
4	8	36.97	1.31	3.07	0.34	16.22	1.19
5	10	34.80	6.70	3.08	0.25	15.79	1.19
6	**12**	**31.37**	**3.23**	**3.63**	**0.52**	**17.72**	**1.22**
7	14	35.25	1.53	4.95	0.67	17.02	1.21
8	16	40.10	0.69	6.03	0.40	16.47	1.20

* Sample with most suitable properties is written **in bold**. ** In relation to the carrier material.

The flowable liquid retention potential (Φ-value) of Fujicalin® mixed with PG was calculated as the ratio of amount of liquid vehicle (10 g) and weight of carrier (40 g) and was equal to 0.25 (Fig. 3).

Mixture of Avicel® PH 101 with propylene glycol

From Figure 4 it is obvious that adding of propylene glycol to Avicel® PH 101 initially decreased the values of angle of slide. Mixtures containing 3-6 g of PG revealed similar values of angle of slide, i.e., about 35.00°. Subsequent adding of propylene glycol increased angle of slide up to 48.67 ± 1.53° (mixture with 8 g of PG), which could be explained by the oversaturation of microcrystalline cellulose with propylene glycol. The value closest to 33° had the mixture containing 6 g of sorbed PG (34.83 ± 0.29°). The angle of repose (31.37 ± 3.23°) and flowability (3.63 ± 0.52 s/50 g) of this liquisolid blend referred to a good character of flow and its values of CI (17.72%) and HR (1.22) corresponded to fair flow properties (34).

Mixing Avicel® PH 101 with an increasing amount of PG implied that adding of a liquid vehicle initially had a negative effect on mixtures' angle of repose. The angle of repose values (Table 8) increased after adding PG up to 36.97 ± 1.31° (blend containing 4 g of PG) and then, after further adding of PG decreased to 31.37 ± 3.23° (6 g of PG). However, subsequent adding of PG increased angle of repose up to 40.10 ± 0.69° again (8 g of PG). The final deterioration of flow properties could have been caused by the oversaturation of carrier particles with PG.

Evaluation of flowability implied similar phenomenon as the angle of repose. Initially, the measured values decreased and then after adding more propylene glycol increased again. The lowest value of flowability (3.07 ± 0.34 s/50 g) showed the liquisolid powder containing 4 g of PG.

Compressibility index and Hausner ratio implied the same tendency as flowability and angle of repose. Their values corresponded to powders with fair/passable flow properties (34).

The flowable liquid retention potential (Φ-value) for Avicel® PH 101/PG admixture was calculated as the ratio of amount of liquid vehicle (50 g) and weight of carrier (6 g) and is equal to 0.12 (Fig. 3). This experimentally measured Φ-value is almost similar to those presented in scientific literature for

Avicel® PH 101 (0.16) (42, 45) and Avicel® PH 102 (0.16) (33, 43).

CONCLUSION

In the technique of liquisolid systems formulation, carrier and coating materials play dominant roles in an effort to obtain dry forms of powder from a drug in liquid state, which are suitable for further processing. Previously, it was observed that a high specific surface area and adsorption capacity of carriers and coating materials allowed incorporation of a greater amount of liquid. The evaluation of flowable liquid retention potential should constitute the main parameter when selecting the most suitable carrier/coating material for formulation of liquisolid systems. From the obtained results it could be concluded that 1 g of Neusilin® US2, Neusilin® UFL2, Neusilin® NS2N, Aerosil® 200, Fujicalin® and Avicel® PH 101 can retain 1.00, 0.97, 0.54, 0.04, 0.25 and 0.12 g of propylene glycol, respectively, while maintaining suitable flowing properties for further processing. Experimentally measured values of flowable liquid retention potential implied that there are available carriers with greater absorption capacity in comparison to the most commonly used microcrystalline cellulose. Magnesium aluminometasilicates (Neusilins®) promised a high potential as carriers and coating materials in the preparation of liquisolid systems.

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EVALUATION OF CASES WITH THE USAGE OF COMMERCIALLY AVAILABLE TABLETS IN THE PEDIATRIC FORMULA

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Abstract: Lack of availability, of either the medicinal product intended to be used for children, or such in a dose which is fitting for the individual child's needs, results in physicians administering medicines meant for the adult. The target of the thesis was to evaluate the cases with the usage of commercially avaible conventional tablet-formulated medicinal products intended for the adult in the pediatric formula. The subjects of the evaluation were the form of the pediatric drug, prepared from commercially available tablets and capsules, as well as the legitimacy of their usage in the treatment of the pediatric population. One hundred and fifty-four prescriptions filled in community pharmacies of Warminsko-Mazurskie Voivodeship in 2011 were chosen. A total of 5805 divided powders in starch capsules were prepared. The prescribing practice included 6 groups of manufactured medicinal products in the form of conventional tablets, containing as follows: anti-hypertensive medicines (ACE inhibitors – enalapril, captopril, ramipril, loop diuretics – furosemide, potassium sparing diuretics – spironolactone, ß-adrenolytics – propranolol, α- and β-adrenolytics – carvedilol), medicines for heart failure (foxglove glycosides – digoxin, methyldigoxin), anti-clotting medicines (acetylsalicylic acid), peristalsis stimulating agents (metoclopramide), antibacterial medicines (furagin), and dopaminergic (carbidopa-levodopa). The only compounded forms ordered by the physicians were divided powders for an internal use. Starch capsules for powder preparation provided the only 'package' for the dose of the compounded powder, which after pouring, solving or suspending in water was administered to children. Such a shift of the form, between an oral tablet and divided powder for an internal use, did not cause a change in the method of administration. The information on indications and the way of dosage for children, inserted in the Summary of Product Characteristics, enables the administration which follows the registered indications, despite the shortage of an appropriate dose in the pharmaceutical market. In contrast, an absence of the information regarding the indications and a dosage for children in the Summary of Product Characteristic, results in an off-label administration, in case of a child.

The pharmacotherapy of the child population, which is non-homogenous, occasionally requires the usage of medicinal products initially registered for adults. Enabling the dosage of a manufactured pediatric medicinal product, meant for various body weights, would result in producing many different doses of the medicine formulation. As following, the technological development, the registration process and the multi-dose manufacturing, appear as non-profitable for the manufacturer (1). This is a reason for many medicines, efficient in child treatment, remaining unavailable in an appropriate dose and formulation. Lack of availability, of either the medicinal product intended to be used for children, or such in a dose which is fitting for the individual child's needs, results in physicians administering medicines meant for the adult.

As an example of such practice, the administration of a manufactured tablet in a dose for adults, requires its adjustment to the child's needs, and the splitting of it into smaller portions. Commonly practised is splitting a tablet into two or four parts by the patient or their carer.

Tablets containing perforations are especially meant for such a case. The method, however, is not wholly precise and should be practised neither with strong agents and a narrow therapeutic index, nor with tablets possessing a notably small diameter. Each part of the split tablet may have a different mass, which is in turn connected to specific, albeit varying, contents of the agent.

Another method aimed at adjusting the dose to the child's needs is that of preparing divided powders for an internal use, compounded from conventional tablets. The aforementioned are prepared based on the doctor's prescription, by authorized individuals, in the pharmaceutical conditions such as the formula room, employing usage of the appro-

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priate scales and utensils. A correct calculation of the amount of tablets to be used for the powder is a vital question. Such a calculation is achieved in relation to a declared amount of the agent in a single tablet. Medicinal substances can be blended with neutral excipients, equipped with filling properties. An increase in the powder mass results, as the used medicinal substance is applied in very small doses. The ready powders are then placed in starch or hard gelatin capsules, as well as in paper sachets. Such a method, however, cannot be used to split modified release tablets. The situation in question could lead to the change of the absorption site and the bioavailability of the medicinal substance.

The following way of the dose adjustment to pediatric needs is solving or suspending the substance (powdered tablet or the content of a capsule) in either a solvent or a dispersing solution.

This is how we achieve liquid forms of drugs which seem to be the most appropriate oral formula addressed to the new-born, infants and younger children. The pharmaceutical market currently offers ready liquid bases, featured with various flavors acceptable to the child's palate.

The disadvantage of this form is a low physicochemical stability of the medicinal substance; a higher, compared with powders, probability of formula incompatibility occurrence; and the growth of microbes in the aqueous surrounding.

Each medicinal product to be marketed in the EU should have complete and up-to-date information before obtaining marketing authorization. This includes the information for patients (Patient's Leaflet) as well as the information for healthcare providers (Summary of Product Characteristics) and labeling (2). Such are the registration documents of the medicinal product manufacturer, necessary to its marketing.

The Summary of the Product Characteristics contains i.a. the following information: the name of the medicinal product; its qualitative and quantitative composition, pharmaceutical form, and therapeutic indications; its posology, and the method of administration for adults and, where necessary, for children (3). The dosage is specified for each method of administration. The European Comission recommends that if the product is indicated in the pediatric population, posology recommendations should be given for each of the relevant subsets. The age limits should reflect the benefit-risk assessment of the available documentation for each subset. If the product is indicated in children and no adequate pediatric formulation can be developed, detailed instructions on how to obtain an extemporaneous preparation should be included in the section dedicated to special precautions for the disposal of a used medicinal product, with a cross-reference in the section for posology and method of administration (4).

The target of the thesis was to evaluate the cases with the usage of commercially available conventional tablet-formulated medicinal products intended for the adult in the pediatric formula.

The subjects of the evaluation were the form of the pediatric drug, prepared from commercially available tablets and capsules, as well as the legitimacy of their usage in the treatment of the pediatric population.

EXPERIMENTAL

The study consisted of the analysis and the evaluation of the compounded drug prescriptions, filled in community pharmacies of Warminsko-Mazurskie Voivodeship in 2011.

The preliminary study embraced 1699 pediatric prescriptions, filled in sixteen community pharmacies. For a further evaluation, the prescriptions with the usage of manufactured tablets meant for adults were chosen. One hundred and fifty-four prescriptions met the above criteria, which constituted 9% of all pediatric prescriptions. One hundred and fifty-four compounded drugs in the form of divided powders for an internal use, in the number placed between 20 and 40 per script, were a result of the aforementioned prescriptions. A total of 5805 divided powders in starch capsules were prepared. The prescriptions for children, containing manufactured tablets, were filled in six pharmacies. This constituted 37.5% of all pharmacies embraced within the study.

RESULTS

Based upon the body of the actual prescription, the authorized personnel, masters of pharmacy or pharmaceutical technicians, prepared divided powders for an internal use.

The preparation of the powders utilized technological processes such as crushing the tablet or pouring the content of a hard capsule and powdering in a mortar; thorough blending with an excipient; proper weighing; and finally, placing into starch capsules with the diameter of 14 mm (No. 2) or of 16 mm (No. 3).

The amount of the excipient used as filler was estimated depending upon the preference of the actual person compounding the medicine. Most

commonly it occurred to hesitate between 0.1 and 0.2 g of dextrose or lactose per unit of powder. The next step took place already in the household conditions, and consisted of emptying the starch capsules from the mixture powder by the children's carers, and stirring or solving it in a small amount of water or milk, and then administering it orally to a child.

The prescribing practice included 6 groups of manufactured medicinal products in the form of conventional tablets, containing as follows: antihypertensive medicines (ACE inhibitors – enalapril, captopril, ramipril, loop diuretics – furosemide, potassium sparing diuretics – spironolactone, β-adrenolytics – propranolol, α- and β-adrenolytics – carvedilol), medicines for heart failure (foxglove glycosides – digoxin, methyldigoxin), anti-clotting medicines (acetylsalicylic acid), peristalsis stimulating agents (metoclopramide), antibacterial medicines (furagin), and dopaminergic (carbidopa-levodopa). Additionally, according to the doctor's order, one of the two excipients were subsequently added to each powder: lactose or dextrose.

The thesis gives both, the analysis and estimate of the following data: the place of the prescribing practice, the doctor's speciality, the patient's age, the content of the compounded drug, its amount and its form. Table 1 shows which of the substances the evaluated amount of pediatric powders contained.

In some patients a few substances were applied at the same time. Each of them was prescribed separately and placed in separate starch capsules. The following combinations of the medicinal substances were reported:

- captopril, carvedilol, spironolactone, β-methyldigoxin
- enalapril, propranolol
- digoxin, captopril, spironolactone
- captopril, spironolactone

The children's ages varied from 2 weeks up to 4 years. The prescribing practice were applied by physicians of the specialities as given: pediatrician, cardiologist, neo-natologist, family doctor, pulmonologist, general practitioner and the non-speciality physician delivering their service within the hospital ward, the outpatient clinic and the hospice home care.

In all studied cases, the same formula of the compounded drug was prepared – divided powders for an internal use in starch capsules, in the number between 20 and 40. For preparation of the compounded drugs were used manufactured medicinal products - ordinary, non-modified release tablets. Moreover, there were used hard capsules filled with a medicinal substance and excipients, as well as hydrophilic membrane tablets – helpful with swallowing and covering up an unpleasant flavor, in case of the latter.

The recommended dosages of active medicines were contained in the Summary of Product Characteristics for each of the following: Captopril Jelfa (2008), Captopril Polfarmex (2008), Enarenal (2007), Axtil (2009), Spironol (2010), Verospiron

Table 1. The occurrence of the medicinal substance in the evaluated amount of compounded pediatric powders.

Medicinal substance	Amounts of powders	% powders (n = 5.805)
Captopril	1870	32.21%
Carbidopa-levodopa	700	12.06%
Spironolactone	660	11.37%
Carvedilol	520	8.96%
β-Methyldigoxin	440	7.58%
Acetylsalicylic acid	365	6.29%
Propranolol	360	6.20%
Enalapril	300	5.17%
Metoclopramide	200	3.45%
Ramipril	160	2.76%
Digoxin	120	2.07%
Furosemide	80	1.38%
Furagin	30	0.52%

(2009), Furosemidum (2008), Propranolol (2008), Carvedilol (2011), Digoxin Teva (2008), Bemecor (2008), Acesan (2010), Metoclopramid (2008), Furaginum (2008), Nacom mitte (2008).

The outcomes of the prescription analysis referred to the ordered medicinal substance; the child's age, the way of dosage of the compounded drugs and the medicinal products used for their preparation, are shown in Table 2.

Hypotensive medicines

Amongst ACE inhibitors only captopril is recommended by the manufacturer to be used with infants and children. Admittedly, there is no manufactured medicinal product in an appropriate dose; the producer, however, gives a thorough description of the pediatric dosage in the registration documents. Captopril is recommended for children with an initial dose of 0.3 mg/kg b.w. and in case of children with kidney dysfunction, preterm infants, newly born and infants with an initial dose of 0.15 mg/kg b.w..

The recommended dosage of enalapril is 2.5 mg for children with body weight between 20 and 50 kg. The medicine is not recommended for the new-born, children and young people with

Table 2. The most frequently prescribed active substances including pharmacological groups, child's age, dosage and commercial medicinal products used to prepare analyzed powders.

Active substance	Child's age	Dosage	Medicinal product used
Hypotensive drugs			
Enalapril	1 - 28 months	0.1-0.65 mg 2 times daily 1 or 2 powders 3 times daily	Enarenal 5 mg × 30 tab.
Captopril	2 – 48 months	1, 2, 3 mg 1, 2 or 3 times daily	Captopril 12.5 mg × 30 tab.
Ramipril	5.5 – 8 months	0.3 mg 1 or 2 times daily	Axtil 2.5 mg × 30 tab.
Furosemide	18 months	2.5 mg 2 times daily	Furosemid 40 mg × 30 tab.
Spironolactone	2 – 12 months	2.0-3.0 mg 1 or 2 times daily	Spironol 25 mg × 20 tab., Verospiron 50 mg × 30 cap.
Propranolol	6 – 12 months	3.0 mg 3 times daily	Propranolol 40 mg × 50 tab.
Carvedilol	2 - 7 months	0.1 or 0.2 mg 2 or 3 times daily	Carvedilol 6.25 mg × 30 tab.
Cardiac drugs			
Digoxin	2 – 3 months	0.015 mg 2 times daily	Digoxin 0.1 mg × 30 tab.
β-Methyldigoxin	2 – 7 months	0.01 mg 2 times daily	Bemecor 0.1 mg × 30 tab.
Anti-clotting drugs			
Acetylsalicylic acid	6 – 17 months	15 mg once daily	Acesan 30 mg × 60 tab.
Peristalsis stimulating drugs			
Metoclopramide hydrochloride	3 years 6 months	2.5 mg 2 times daily	Metoclopramid 10 mg × 50 tab.
Antibacterial drugs			
Furagin	10 months	10 mg once daily	Furaginum 50 mg × 30 tab.
Antiparkinsonism drugs			
Carbidopa-levodopa	4 years	10 mg 2.3 times daily	Nacom mitte

glomerular filtration rate (GFR) < 30 mL/1.73 m^2. In the studied cases the medicine was given to children of the age from 2 weeks up to 28 months. The dosage was determined by the manufacturer based upon the body weight of the child. In contrast, the information about the child's weight was not given in the prescription which is the basis for preparation of a compounded drug. The manufacturer of ramipril does not recommend the usage in cases of children and young people below the age of 18 in relation to the lack of enough safety and efficacy data.

In the evaluated prescriptions for preparation of a compounded drug, one used spironolactone of two manufacturers. The descriptions from the registration documents of both vary subtly as for the usage in children. One of the producers recommends the usage for children and young people below 18 with a saturating dose of 2–3 mg/kg b.w. in 1-4 divided doses and one maintaining dose of 1-1.5 mg/kg b.w. The medicine is possible to crush and then administer with fluid. While the other manufacturer restricts only to a short description: 3.0 mg/kg b.w. once or in two divided doses.

According to the registration documents, furosemide cannot be applied in children who are not capable of swallowing a tablet. In this case, the dosage places between 1 mg and 3 mg/kg b.w. once every 24 h. One must not apply the doses larger than 40 mg per 24 h independently from the child's body mass.

α-Adrenolytics as well as α- and β-adrenolytics were represented by propranolol and carvedilol. The former is recommended for children above 6 at the dose of 20 mg 2-3 times daily. The manufacturer of the latter shows the lack of experience in usage of the drug in case of children and young people.

Cardiac drugs

Digoxin is recommended to be used with infants and children. The manufacturer did not market the pediatric dose, however, they nevertheless described thoroughly the way of dosage in the registration documents - for infants and children up to 2 years old a saturating dose 20-35 μg/kg b.w., with a maintaining dose 5-9 μg/kg b.w., for children 2-12 years old a saturating dose of 10–15 μg/kg b.w., with a maintaining one 3-5 μg/kg b.w..

It was observed that in the case of the other child the doctor ordered β-methyldigoxin. The producer of this medicine in the registration documents does not give any information referring to the safety or unsafeness of the usage with children. The dosage of this drug is related to the adult group only.

Anti-clotting drugs

Acetylsalicylic acid should not be used for children up to 12 years in case of viral infections because of the possibility of Reye's syndrome occurrence.

Peristalsis stimulating drugs

Metoclopramide hydrochloride should not be applied for children below 15 years of age.

Antibacterial drugs

The indications for the application of furagin refer to children over 2 up to 14 in a dose of 5 mg to 7 mg/b.w. 24 h in 2-3 divided doses. A tablet can be crushed and stirred with milk. In case of children below 2 the dose has to be estimated directly by the physician.

Antiparkinsonism drugs

Complex medicines consisting of carbidopa and levodopa are not recommended for patients below 18 years since there is not sufficient data regarding either the safety or the efficacy in infants and children.

DISCUSSION

One of the vital issues of the thesis was verifying and evaluating the way the pharmaceutical market responds to the needs of pediatric pharmacotherapy when faced with the shortage of appropriate forms and doses of a medicinal product. Deduced from the literature the usage of such drug forms as suppositories and per rectum fluids is recommended for the new-born and infants. Starting from the one-month-old liquid forms are preferable, along with those of tablets, granulates or powders which can be administered with drinks or food (1). The researches conducted in 16 European countries in 2003 showed that the methods of preparing a pediatric form and dose varied depending upon the country where the compounded drug was prepared. In Denmark, England, Ireland, Norway and Sweden, liquid forms seem to be preferable. The majority of such are drops, solutions, suspensions, emulsions and sirups (5, 6).

There are still many countries in Europe where pharmacists prepare their own sirups and suspending vehicles due to low cost, local availability, and the policy. Contamination is more likely to occur in a poorly-preserved preparation when a single storage container is placed in and out of refrigeration for multiple sampling during the intended in-use shelf life of an extemporaneous preparation. The use of

commercially available vehicles is recommended (7). For oral liquids in European formulary practice, the universal, commercial suspending vehicles are used (for example: Ora®-products). Such consist of suitable preservatives (methylparaben, potassium sorbate) and flavors, and allow to spare time for preparing formulation, to mask an unpleasant taste and to provide the conditions which make the preparation more stable (8).

There are several studies concerning the stability of medicinal substance in a liquid vehicle which were conducted in the USA and in Europe. Oral pediatric liquids with propranolol, prepared from tablets in a simple sirup with sorbitol and glycerol with no preservative remained stable for at least 35 days, stored either at 25°C or 4°C. Oral pediatric liquids with enalapril maleate, prepared from tablets in a raspberry sirup or 85% orthophosphoric acid solution, remained stable for at least 30 days, stored at 25°C or 4°C. All suspensions contained methyl hydroxybenzoate 0.2% as a preservative (8, 9).

Even if no microbial or fungal growth was observed in the non-preserved medium, there is a lack of any reliable evidence regarding its microbiological stability. Therefore, a storage time exceeding 14 days should be avoided (8, 9).

In Belgium, Croatia, France, Sweden and Switzerland, hard gelatine capsules are a preferred form, whose structure consists of two cylindrical parts, both with rounded ends (5, 6). A powdered substance is placed in one and closed with the fitting other. These capsules are usually intended to be swallowed as a whole; however, in case of infants and young children, one should open a capsule and having its content mixed with a small amount of liquid, administer as a drink (6). In Italy, Finland, Scotland and Portugal, the most commonplace are divided powders (5, 10). Such are packed in paper sachets. All substances, after being powdered and weighed, are put into small, folded paper bags, every single one of which is treated as a unit dose of the drug. The medicine is administered after solving the content in water, milk or other fluid.

In the group of prescriptions, reviewed in the thesis, the only form of a compounded drug prescribed by physicians were divided powders for an internal use. Such powders were prepared with the usage of starch capsules. This form is intended to be swallowed as a whole, which makes it inappropriate for infants and young children. In the referred cases, the aforementioned had to be taken as a specific unit dose 'package' in order to be administered to children, after mixing with drink as a liquid suspension or a solution.

In the actual pharmaceutical formula, the used medicinal products were only non-modified release tablets and plain capsules. After micronization of a tablet, or the content of a hard gelatine capsule, the powders were stirred with an excipient, lactose or dextrose.

The size of the crushed grains of the powder plays a crucial role in the stability of the medicinal substance, among others upon its photochemical stability. The smaller the size of the grains, the more rapidly the photo-degradation rate constant grows. Facing that fact, the usage of lactose was desirable. Lactose is easily prone to a free radicals attack. Most likely, it acts as a free radicals transmitter in order to inhibit the medicinal substance degradation (11).

It should be noted that pharmacy-compounded, divided powders, enabled the application of the pediatric doses which are not manufactured by the pharmaceutical industry. The shift of the form of medication did not change the method of administration, as in all the cases the drug was to be taken orally.

The other analyzed issue was the legitimacy of the usage of the medicinal products being a subject of the study in the treatment of the child population. The manufacturers of medicinal products are obliged in the registration process to prepare the Summary of the Product Characteristics intended for medical personnel, as well as the patient's leaflet, both containing i.a. indications, precautions and the way of dosage of the product. In case of those medicinal products which are in the registration documents intended and precisely described as for the dosage for children, there is no doubt pertaining to their usage. This refers to medicinal products containing captopril, spironolactone, digoxin and furagin. Such group may also include furosemide. The manufacturer does not, however, recommend the drug in case of children who have problems with swallowing a tablet. Although it does appear more profitable for the patients to administer the medicine, in the form of divided powders that are processed into a liquid form in the household conditions by the child's carer. In such a situation, there is no barrier with swallowing the tablet. The appropriate dosage for children was determined by the manufacturer as ranging from 1 to 3 mg/kg b.w.

The problem occurs in case of the pediatric usage of those medicinal products which are not indicated for children, or do not have an appropriate dose. The treatment beyond the indications given in the Summary of the Product Characteristics is named as 'off-label'. Prescribing an 'off-label' medicine results in accepting responsibility for the effect

of its action. A physician can prescribe an 'off-label' drug beyond the medical experiment and clinical trials procedures only if the aforementioned physician does not expect the possibility of such constituting an illegal act, or does not intend the extension of the medical knowledge (to discover or to prove the effect of the drug), and the efficacy of the hitherto applied methods appears to be insufficient (12).

The usage of off-label drugs in the pediatric population is known worldwide. The off-label medicines are applied first and foremost during hospital treatment. For instance, based upon the studies carried out in France among 989 hospitalised patients, it was revealed that 56% of children had one or more off-label status medicines prescribed (13).

Whereas, the Brazilian research conducted upon 61 patients had demonstrated that 27.7% of the prescribed drugs for infants were off-label medication (14). Parallel with these was the Palestinian study, where 35.3% of 387 hospitalized children had the drugs of the same as the aforementioned status prescribed (15).

The preparation of off-label drugs in community pharmacies is most commonly connected with post-hospital treatment and a necessity for continuing hospital-ordained pharmacotherapy.

In the literature, there are four cases of drug usage beyond the strictly registration-adhered indications (16):

1. The usage of a medicinal product in a way which is not mentioned in the registration documents.
2. The usage of a drug following the registered indications in patients for whom there is no dosage determined.
3. The usage of a drug in an indication that is not mentioned in the registration documents, but of which there is reliable data warranting its safety and efficacy.
4. The usage of a drug in a new indication which is not mentioned in the registration document, but of which there is scientific-based evidence warranting its efficacy and safety.

Enalapril (3 cases), propranolol, metoclopramide and acetylsalicylic acid were applied in the patients for whom there is no dosage determined. The registration-related indications of enalapril dosage refer to the child's body weight and recommend the dose of 2.5 mg for a child of 20–50 kg b.w. The data in the prescription give only the child's age, and they should not be compared with the child's body weight; however, the assumption of a two-week- or one-month -old child whose body weight is at least 20 kg does not seem to be acceptable. In the registration documents the manufacturer did not take into consideration children with the body weight below 20 kg. Propranolol is recommended for children above the age of 6. The drug, however, has been administered to a patient between 6 and 12 months old. Metoclopramide should not be used with children below the age of 15. The medicine, though, was administered to a 4-year-old child. The registration documents of acetylsalicylic acid do not contain the dosage for children. The other remaining substances i.e., ramipril, carvedilol, methyldigoxin and carbidopa-levodopa do not have registration indications applying to the treatment of children.

In the aforementioned situations, the medicines which were administered to children had an 'off-label' status. In cases of some indications, the Polish Ministry of Health accepts the usage of 'off-label' drugs and refunds their cost. Since 2012, the off-label medicines have been mentioned in the list of reimbursed drugs, including the range of their out-of-registration indications which are embraced with the reimbursement. Amongst the off-label drugs accepted by the Ministry of Health for children below 18 are those described in the thesis: carvedilol, ramipril, carbidopa-levodopa, propranolol and enalapril (17).

CONCLUSION

Ordaining and preparing compounded drugs in community pharmacies created the possibility of adjusting the doses of medicinal substances, contained in manufactured medicinal products intended for the adult, to individual needs of the children. The only compounded form ordered by the physicians were divided powders for an internal use. The ordination of any other pediatric form was not observed. Starch capsules for powder preparation provided the only 'package' for the dose of the compounded powder, which after pouring, solving or suspending in water was administered to children. The addition of lactose to the content of the compounded drug may contribute to the growth of photo-stability of the medicinal substance received *via* micronization of a ready medicinal product. Such a shift of the form, between an oral tablet and divided powder for an internal use, did not cause a change in the method of administration. The information on indications and the way of dosage for children, inserted in the Summary of Product Characteristics, enables the administration which follows the registered indications, despite the shortage of an appropriate dose in the pharmaceutical market. In contrast, an absence of the information regarding the indications and a

dosage for children in the Summary of Product Characteristic, results in an off-label administration, in case of a child.

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RELEASE AND MUCOADHESION PROPERTIES OF DICLOFENAC MATRIX TABLETS FROM NATURAL AND SYNTHETIC POLYMER BLENDS

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Abstract: The delayed release and mucoadhesive properties of Cedrela gum and hydroxypropylmethylcellulose blend in diclofenac sodium tablet formulations were evaluated. Tablets were prepared by direct compression and the crushing strength and detachment force were found to increase from 74.49 ± 1.22 to 147.25 ± 2.57 N and 0.302 ± 0.36 to 1.141 ± 0.05 N from low to high level of polymers, respectively. The release kinetics followed Korsmeyer-Peppas release and the *n* varied between 0.834 and 1.273, indicating that the release mechanism shifts from Fickian to super case I (anomalous release). The drug release profile fits a pulsatile-release pattern characterized by a lag time followed by a more or less rapid and complete drug release. The Cedrela gum-hydroxypropylmethylcelluse blend tablets delayed diclofenac release for 2 h and sustained the release for 12 h. The polymer blend delayed drug release in the 0.1 M HCl simulating gastric environment and subsequent release pH 6.8 phosphate buffer.

Keywords: Cedrela gum, delayed release, ducoadhesion matrix, polymer blend, release mechanism

Mucoadhesion has been defined as interfacial force interactions between polymeric materials and mucosal tissues. Significant attention has been paid to the design of novel drug delivery systems with ability to prolong the residence time of dosage forms as well as sustain drug release and consequent bioavailability (1-4). Various routes of administration such as ocular, nasal, buccal, vaginal and rectal, make mucoadhesive drug delivery systems an attractive and flexible dosage form.

While several synthetic and natural polymers have been investigated extensively for this purpose (2, 5), the use of natural polymers for pharmaceutical applications is attractive because they are readily available and economical, non-toxic, potentially biodegradable and with few exceptions, also biocompatible. Also, plant resources, if cultivated or harvested in a sustainable manner, can provide a renewable supply of raw materials. Natural polymers have been successfully employed to formulate solid, liquid and semi-solid dosage forms and are specifically useful in the design of modified release drug delivery systems (5, 6).

Many synthetic polymers such as polyacrylic acid (PAA), polymethacrylic acid, cellulose derivatives, polyethylene oxide have been used as mucoadhesive drug carriers. However, these are associated with undesirable mucosal irritation and hence, the need for the development of natural polymers as bioadhesive drug delivery systems (7, 8). Controlled release drug delivery technology minimizes the frequency of administration by keeping the drug in therapeutic window for a longer period, improves patient compliance and reduces drug wastage by optimizing the efficacy of drugs (9, 10). However, controlled release technology generally is inadequate and incapable of increasing gastric resident time of drugs (11, 12).

Cedrela odorata (Meliaceae) is a widely distributed tropical plant and produces a clear gum. The polysaccharide isolated from the *C. odorata*, contains galactose, arabinose and rhamnose as neutral sugars and uronic acid residues. These sugar acids are represented by glucuronic acid and its 4-*O*-methyl derivative. The cationic composition of the ash showed the presence of calcium and magnesium predominantly (13). The flow behavior of *C. odorata* dispersion under steady shear is highly non-Newtonian and is characterized by the lack of a low-shear limiting Newtonian viscosity plateau even at very low shear rates (14).

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Cedrela gum has been found to be effective as a suspending agent (15), a carrier for theophiline in microcapsule formulation (16) and as a binder in tablet formulations at relatively low concentrations with potential bioadhesive properties (17). Cedrela gum hydrates quickly and hence cannot form a strong gel to retard drug release, while hydroxypropyl methylcellulose forms firm gel but do not hydrate quickly (18, 19). Further, while the mucoadhesive properties of HPMC have been found to be only moderate, the gum displays significant adhesive potential in a tablet formulation (17). Hence, a blend of HPMC and Cedrela gum is being investigated in order to overcome the limitations of the individual polymers. The initial drug burst release observed with formulations incorporating HPMC would be controlled by the strong binding properties of the natural gum while the subsequent drug release and matrix integrity maintained by the firm gel formed by HPMC.

Diclofenac sodium (DS), a widely used non-steroidal anti-inflammatory drug that exhibits anti-rheumatic, analgesic, osteoarthritis, and anti-pyretic activities, was chosen as the model drug. It has a short half-life in plasma of one to two hours.

MATERIALS AND METHODS

Materials used in this study include diclofenac sodium (Unique Chemicals, Gujarat, India), Cedrela gum (obtained from the incised trunk of *Cedrela odorata* (Meliaceae) tree, Botanical Gardens, University of Ibadan, Ibadan, Nigeria, hydroxypropyl methylcellulose (Methocel® K100M, Colorcon, UK), Tablettose (Meggle Pharma, Germany), Aerosil (Uitgest, Holland), magnesium stearate (R&M Chemicals, Essex, UK). Materials were used as received. Other reagents were of analytical grade.

Preparation of gum

Cedrela gum was extracted from the incised trunk of *Cedrela odorata* from the Botanical Garden, University of Ibadan (Ibadan, Nigeria) and authenticated at the Department of Botany Herbarium, University of Ibadan (UIH-22378), and purified using previous methods (17). Briefly, the exudate was hydrated in 0.5 : 95.5 (v/v) $CHCl_3$/water mixture for five days with intermittent stirring; extraneous materials were removed by straining through a muslin cloth. The gum was precipitated from solution with absolute ethanol. The precipitated gum was filtered, washed with diethyl ether, and then dried in hot air oven at 40°C for 18 h. The gum was pulverized using a laboratory blender, sieved and the size fraction < 170 µm was used for the study.

Formulation design and matrix tablet preparation

The formulation design for the matrix tablets is given in Table 1. Cedrela gum was evaluated at 10, 30 and 50 mg while HPMC K100M was evaluated at 10, 20 and 30 mg in the tablet formulations.

Study of interaction between Cedrela gum and diclofenac sodium

Fourier transform infrared (FTIR) spectroscopy (on Model 2000 Perkin Elmer Spectroscopy, USA apparatus) was carried out to check the compatibility of the drug and excipients in the final formulation. The IR spectra of the samples were obtained using KBr discs that were prepared with hydraulic press after careful grinding of a small amount of each sample with KBr. The spectral width was 400–4,000 cm^{-1}. Each spectrum was acquired by performing 32 scans.

Preparation of matrix tablets

Matrix tablets were produced by weighting, screening, and mixing the excipients through a 40-

Table 1. Formulation design for diclofenac sodium matrix tablets (in mg).

Ingredient	F1	F2	F3	F4	F5	F6	F7	F8	F9
Diclofenac sodium	200	200	200	200	200	200	200	200	200
Cedrela gum	10	30	50	10	30	50	10	30	50
HPMC	10	10	10	20	20	20	30	30	30
Tabletosse	176	156	136	166	146	126	156	136	116
Aerosil	2	2	2	2	2	2	2	2	2
Magnesium stearate	2	2	2	2	2	2	2	2	2
Total (mg)	400	400	400	400	400	400	400	400	400

mesh sieve, to which the active ingredient was added and mixed thoroughly. Bulk density and tapped density of the powder blend was determined with graduated cylinders according to USP guidelines. Hausner ratio and Carr's index were determined to assess the flow property and compressibility of the powder blend (20). The powders were compressed using a tabletting machine (Manesty Machine Ltd., England) fitted with round, concave faced, 10 mm diameter punches and dies. The compression force was 1 tonne.

Evaluation of tablets

Twenty tablets were powdered individually and a quantity equivalent to 100 mg of diclofenac sodium was accurately weighed and extracted with a suitable volume of pH 6.8 phosphate buffer. Each extract was filtered through Whatman filter paper No. 41 (Whatman Paper Limited, UK) and analyzed spectrophotometrically (Hitachi U2000, Tokyo, Japan) at 276 nm after sufficient dilution.

The matrix tablets were also evaluated for crushing strength using a hardness tester (Erwerka Aapparatebau GmbH, Germany), friability (Erweka Aapparatebau GmbH, Germany), weight variation using analytical balance (Citizen CY 200), and thickness using digital micrometer gauge (Mitutoyo, Japan).

Determination of *ex vivo* mucoadhesive strength

Mucoadhesion testing was conducted *ex vivo* using freshly incised cow intestine from a slaughter house. Measurements were made with a Texture Analyser (TA-XT2i, Stable Micro Systems, Surrey, UK). Each tablet was attached to the base of an aluminium probe (using double-sided adhesive tape) fixed to the mobile arm of the texture analyzer. The tablet was lowered at a rate of 0.1 mm/s until contact with the intestine was made. A contact force of 0.25 N was maintained for 5 min, after which the probe was withdrawn from the intestine at a rate of 0.1 m/s. The peak detachment force (N) was recorded as a measure of bioadhesion (21, 22). Triplicate determinations were made with typically a coefficient of variation (cv) of < 5 %.

Drug release

The *in vitro* drug dissolution study was carried out in 900 mL of 0.1 M HCl at 37.0 ± 0.5°C for the first one hour and pH 6.8 phosphate buffer for 11 h,

Table 2. Micromeritic properties of powder blends of the various formulations.

	Formulation code								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Bulk density (g/cm^3)	0.476	0.435	0.455	0.417	0.455	0.385	0.385	0.370	0.385
Tapped density (g/cm^3)	0.625	0.588	0.625	0.625	0.556	0.526	0.556	0.526	0.556
Carr's index (%)	23.810	26.087	27.273	33.333	18.182	26.923	30.769	29.630	30.769
Hausner's ratio	1.313	1.353	1.375	1.500	1.222	1.368	1.444	1.421	1.444

Table 3. Physical characteristics and bioadhesive properties of matrix tablets.

Formulation code	Diameter (mm)	Thickness (mm)	Crushing strength (N)	Friability (%)	Drug content (%)	Peak detachment force (N)
F1	10.0 ± 0.05	5.26 ± 0.03	74.49 ± 1.22	0.12 ± 0.01	98.42 ± 1.63	0.304 ± 0.24
F2	10.0 ± 0.03	5.27 ± 0.01	85.31 ± 1.43	0.01 ± 0.03	98.03 ± 1.54	0.302 ± 0.36
F3	10.0 ± 0.04	5.20 ± 0.06	113.29 ± 2.21	0.01 ± 0.01	98.51 ± 2.39	1.236 ± 0.13
F4	10.0 ± 0.01	5.30 ± 0.04	90.65 ± 0.14	0.01 ± 0.01	98.45 ± 2.18	0.740 ± 0.04
F5	10.0 ± 0.05	5.28 ± 0.03	147.25 ± 2.57	0.01 ± 0.01	98.83 ± 1.39	1.017 ± 0.08
F6	10.0 ± 0.03	5.28 ± 0.01	109.76 ± 1.32	0.01 ± 0.01	99.26 ± 1.17	0.499 ± 0.05
F7	10.0 ± 0.01	5.29 ± 0.02	97.12 ± 1.54	0.01 ± 0.02	99.62 ± 1.54	0.963 ± 0.02
F8	10.0 ± 0.05	5.28 ± 0.01	103.93 ± 2.11	0.00 ± 0.01	98.58 ± 2.23	0.989 ± 0.03
F9	10.0 ± 0.01	5.21 ± 0.02	132.01 ± 3.27	0.00 ± 0.01	98.79 ± 1.93	1.141 ± 0.05

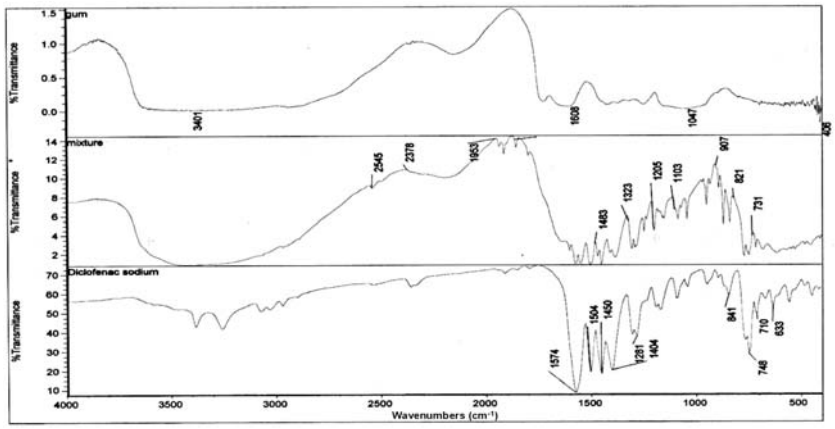

Figure 1. FTIR spectra of Cedrela gum, diclofenac sodium and physical mixture of gum and drug

using USP basket method at a stirring speed of 100 rpm. Samples were withdrawn and immediately replaced with an equal volume of fresh dissolution medium at predetermined intervals. The samples were filtered using a 0.45 µm membrane filter and the amount of drug released was determined using UV spectrophotometer (Hitachi U2000, Tokyo, Japan) at 276 nm.

Mechanism of drug release

Drug release from tablet formulations may follow either zero order kinetics which describes the systems where the drug release rate is independent of its concentration (23), first order kinetics where the release rate is concentration dependent (24), or Higuchi's model in which the release of drugs from insoluble matrix as a square root of time-dependent process based on Fickian diffusion (25). The Hixson-Crowell cube root law describes the release from systems where there is a change in surface area and diameter of particles or tablets (26). However, in order to determine the mechanism of drug release from the formulation, release data may be fitted in Korsmeyer et al. (27) equation (Equation 1):

$$\text{Log } (M_t/M_f) = \text{Log } k + n\text{Log } t \quad (1)$$

This equation describes drug release behavior from polymeric systems. M_t is the amount of drug release at time t, M_f is the amount of drug release after infinite time; k is a release rate constant incorporating structural and geometric characteristics of the dosage form and *n* is the diffusional exponent indicative of the mechanism of drug release (27, 28). For a cylinder shaped matrix the value of n = 0.45 indicates Fickian (case I) release; > 0.45 but < 0.89 for non-Fickian (anomalous) release; and > 0.89 indicates super case II type of release. Case II mechanism refers to the erosion of the polymer and anomalous transport (non-Fickian) refers to a combination of both diffusion and erosion controlled drug release (29).

The mean dissolution time (MDT), was proposed by Möckel and Lippold (30), as providing a more accurate drug release rate that the t_x%. The equation is used to characterize drug release rate from the dosage form and the retarding efficiency of the polymer. Values of MDT can be calculated from dissolution data using the equation:

$$MDT = (n/n + 1)k^{-1/n} \quad (2)$$

where n is the release exponent and k is release rate constant. A higher value of MDT indicates a higher drug retaining ability of the polymer (31).

Statistical analysis

Statistical analysis was carried out using Students' *t*-test and ANOVA, p value lower or equal to 0.05 was considered the limit of significance.

RESULTS AND DISCUSSION

No significant shifts or reduction in the intensity of FTIR bands of diclofenac sodium was observed in the physical mixture with the gum. Characteristic peaks present in the FT-IR spectrum of Cedrela gum and HPMC K100M appeared in the spectra of the physical mixture with diclofenac sodium indicating the absence of any chemical interaction between the drug and the excipients (Fig. 1).

The micromeritic properties of the formulations are given in Table 2. There was no substantial difference in the bulk and tapped densities of all the

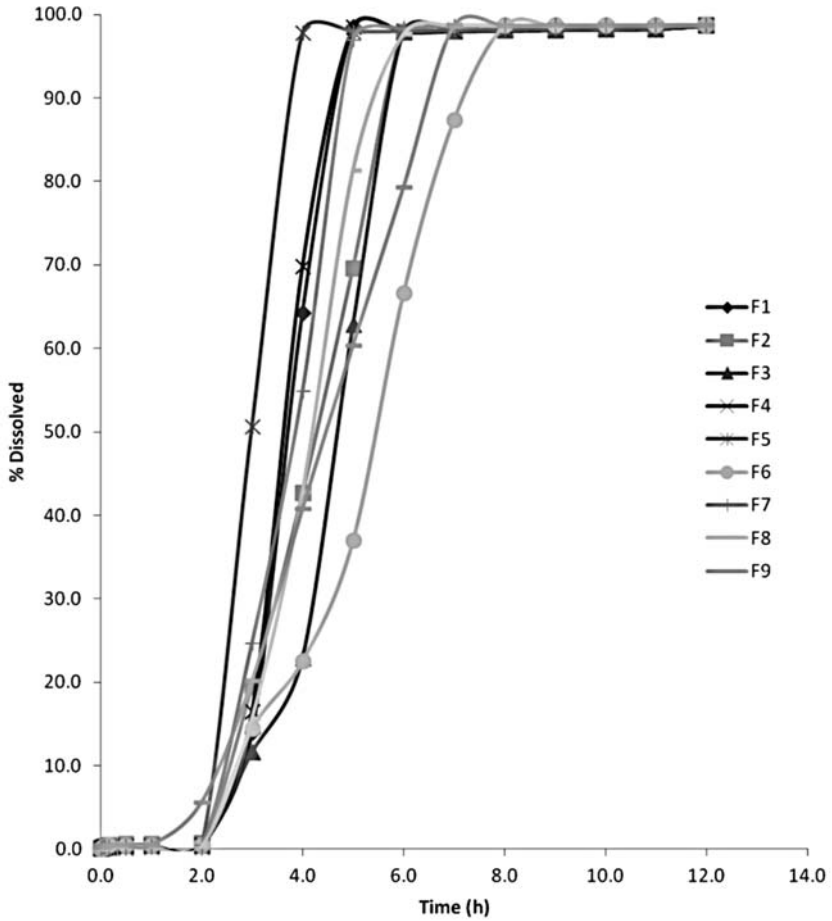

Figure 2. Cumulative release of diclofenac sodium from batches F1 to F9. Each point represents the mean ± SD (n = 3)

formulations. Table 2 shows that compressibility index was highest for batch F4 (containing Cedrela gum/HPMC at ratio 1 : 2) and lowest for F5 (containing Cedrela gum/HPMC at ratio 3 : 2). The flow property was determined by Hausner ratio (1.22-1.50) and Carr's index (18.18-33.33%). Carr's compressibility index is an indication of the compressibility and flowability of a powder, and is also a direct measure of the propensity of a powder to consolidate when undergoing vibration, shipping and handling (6). Also, Hausner's ratio, presented in Table 2, is an indication of the flowability of powders. Formulation F5 had the lowest value (1.2) and hence the highest flowability, while F4 (1.5) had poor flow properties.

The tablets produced from the powder blends had uniform thickness, low friability and a high degree of content uniformity (Table 3). This indicates that the direct compression method is suitable for preparing matrix tablets of DS. The assayed content of DS in the various formulations varied between 98.03 and 99.62% (mean 98.83%) while the friability of the batches complied with British Pharmacopoeia (Table 3).

The crushing strength values, a measure of the hardness of tablets, are presented in Table 3. The hardness of the tablets increased proportionally with the amount of polymers in the formulation due to their binding properties. Formulations containing higher amounts of the natural gum contributed a greater effect to the crushing strength of the matrix tablets than HPMC ($p < 0.001$). This agrees with the previous study by Odeniyi et al. (17) using Cedrela gum as a binder in ibuprofen tablet formulations by wet granulation process. The results show that the bond forming property of the gum is not dependent on process of tablet formulation but increases with availability of binding surfaces provided with increasing polymer concentration. An additive effect could further be observed, as formulation F9

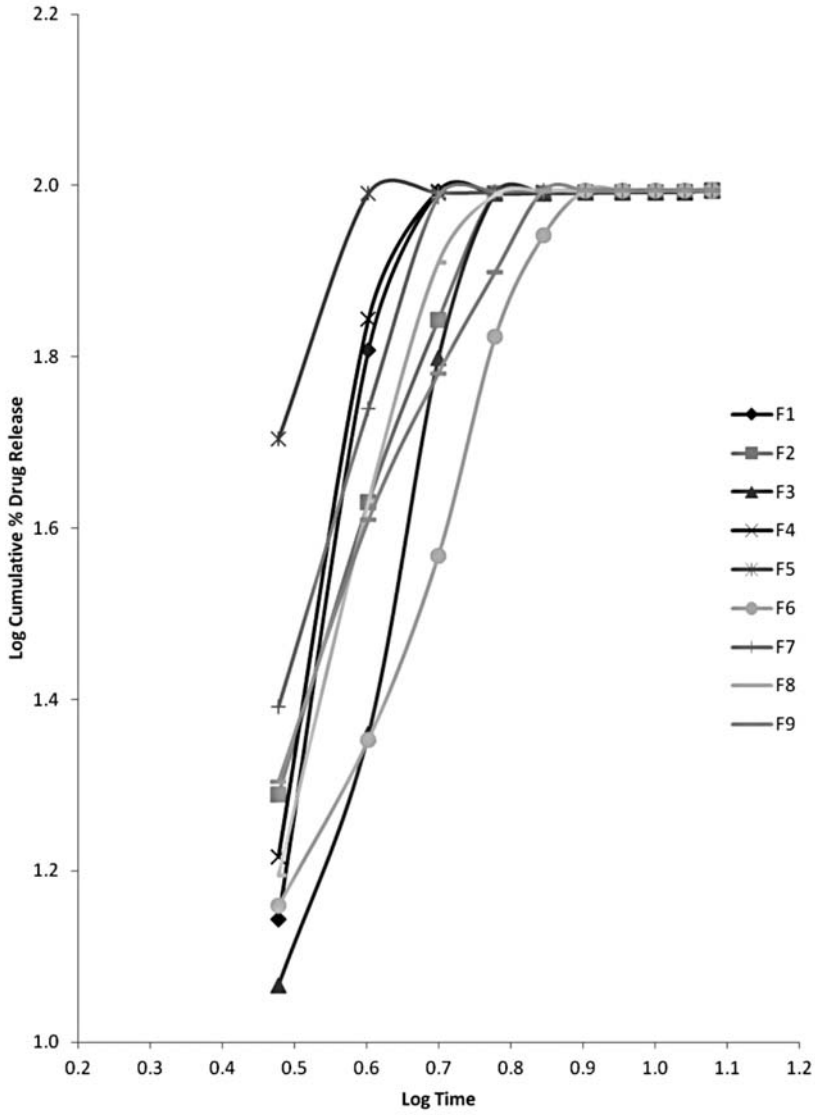

Figure 3. Korsmeyer-Peppas model for mechanism of drug release from matrix tablets

incorporating the highest concentration of both Cedrela gum and HPMC had the highest crushing strength values. Polymer type and amount in a formulation have been demonstrated to affect the crushing strength of tablets (32). This effect has been attributed to the plasto-elastic property of both polymers in the formulation. Cedrela gum was shown to undergo plastic deformation and thereby forced into the interparticulate spaces between the drug particles. This causes an increase in the contact area between the particles thereby forming solid bonds (17, 33).

Dissolution studies results are presented in Figure 2. A lag time of about 2 h was observed for the formulations. This was due to the fact that the first hour of dissolution was conducted in 0.1 M HCl, simulating the stomach. The drug release profile fits a pulsatile-release pattern which is characterized by a lag time followed by a more or less rapid and complete drug release (34). Drug release into the acidic stomach medium is avoided and release into the intestine or colon can therefore be achieved due to the apparent pH-dependent swelling and drug release of the Cedrela gum-HPMC blend. Further, the time taken for 25% (t_{25}), 50% (t_{50}), 75% (t_{75}) and 90% (t_{90}) drug release were obtained from the dissolution plot (Table 4). The lag times obtained from the dissolution curve generally increased with

Table 4. Dissolution parameters of matrix tablets.

Formulation code	t_{25}% (h)	t_{50}% (h)	t_{75}% (h)	t_{90}% (h)	MDT (h)	Lag time (h)
F1	3.358	3.818	4.116	4.257	3.739	2.000
F2	3.023	3.487	5.297	7.585	4.186	2.313
F3	4.000	4.045	4.797	6.893	4.537	2.878
F4	4.000	4.000	4.000	4.010	3.653	2.000
F5	3.000	3.000	3.000	3.134	3.031	1.346
F6	3.425	4.874	7.169	8.936	5.183	2.917
F7	3.000	3.080	4.051	6.343	3.710	2.000
F8	4.000	4.000	4.108	5.446	4.091	2.345
F9	2.014	4.473	7.137	8.806	4.411	2.529

Table 5. *In vitro* release kinetics of matrix tablets.

Formulation code	Zero-order		First-order		Higuchi		Hixson-Crowell		Korsmeyer-Peppas		
	r^2	k_0	r^2	k_1	r^2	K_H	r^2	K_{HC}	N	r^2	K
F1	0.825	10.736	0.866	0.270	0.908	37.716	0.894	0.076	0.012	**0.994**	97.047
F2	0.883	10.408	0.885	0.239	0.946	37.447	0.916	0.067	0.265	**0.970**	60.078
F3	0.875	10.199	0.848	0.220	0.933	38.879	0.882	0.063	0.141	**0.980**	77.441
F4	0.820	10.784	0.869	0.277	0.907	37.924	0.897	0.078	0.017	**0.991**	97.439
F5	0.762	11.110	0.887	0.333	0.876	36.946	0.910	0.094	0.021	**0.999**	93.847
F6	0.923	9.634	0.860	0.188	**0.960**	36.675	0.896	0.054	0.527	0.958	34.867
F7	0.840	10.756	0.881	0.271	0.923	37.770	0.910	0.076	0.158	**0.976**	74.401
F8	0.868	10.512	0.874	0.245	0.935	37.990	0.906	0.069	0.070	**0.989**	87.699
F9	0.918	10.207	0.903	0.224	0.965	37.356	0.934	0.063	0.867	**0.994**	13.663

polymer concentration. Higher values were observed to correlate with increase proportion of the polymers in the formulations. At higher polymer loading, the viscosity of the polymer gel increases and this results in ineffective diffusion of the drug (35). Cedrela gum at highest concentration (formulation F6) modulated the release profile giving a sigmoidal curve when compared with other formulations. With further increase in HPMC concentration, a thicker gel is formed, which inhibits water penetration and resulting in significant increase in t_x values.

MDT values are also given in Table 4. They ranged between 3.03 – 5.18 h and increased with polymer loading. A positive correlation was observed (0.873) between MDT and t_{90}.

The dissolution data were best fitted to the Korsmeyer-Peppas equation (Fig. 3) with correlation coefficient of 0.96 – 0.99. The *n* values for all the formulations ranged from 0.01 to 0.87 (Table 5). This shows that the release mechanism for all the formulations, except F6 and F9, was Fickian (case I) release. Solute diffusion, polymeric matrix swelling and material degradation have been suggested to be the main driving forces for solute transport from drug containing polymeric matrices. Fickian diffusion, based on Fick's law of diffusion, refers to the solute transport process in which the polymer relaxation time is much greater than the characteristic solvent diffusion time. This mechanism is associated with solute concentration gradient, the diffusion distance, and degree of swelling (36). It has been shown that the presence of monovalent ions like Na^+ or K^+ tend to reduce swelling and increase rate of drug release from matrix tablets (36). However, for formulations incorporating the high amounts of Cedrela gum and high and intermediate amounts of HPMC K100M (F6 and F9) (Table 5), the *n* values were > 0.45 but < 0.89 indicating anomalous transport (non-Fickian) which is a combination of both diffusion and erosion controlled drug release (29).

The values of peak detachment force, which is a measure of mucoadhesive strength, of the tablet formulations are presented in Table 3. The values range from 0.302 to 1.141 N. Mucoadhesive strength was observed to increase with amount of polymer in the tablet formulations ($p < 0.05$). The highest value was observed in the formulation F9 incorporating the highest levels of the two polymers. Increasing the amount of polymers provided more adhesive sites and polymer chains for interpenetration with mucin. This will consequently increase the adhesion strength of the formulations (37). The high adhesion value obtained with the polymers in this case could be due to increase in hydrogen bonding effects (38).

CONCLUSION

Mucoadhesive and delayed release matrix tablets of diclofenac sodium were obtained by using a blend of Cedrela gum and hydroxypropyl methylcellulose (Methocel® K100M). The inclusion of hydroxypropyl methylcellulose, in the matrix tablets of diclofenac sodium led to increase in the mechanical, release retarding and mucoadhesive properties of Cedrela gum in the matrix tablets. Drug release was pulsatile-like and was dependent on the amount and type of matrixing agent The kinetics of drug release was explained by Korsmeyer-Peppas model. A blend of polymers by varying the proportions of Cedrela gum and hydroxypropyl methylcellulose could be used to formulate targeted and delayed release tablets.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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EVALUATION OF ALGINATE MICROSPHERES WITH METRONIDAZOLE OBTAINED BY THE SPRAY DRYING TECHNIQUE

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Abstract: In the present study, nine formulations (F1-F9) of alginate microspheres with metronidazole were prepared by the spray drying technique with using different drug : polymer ratio (1 : 2, 1 : 1, 2 : 1) and different sodium alginate concentration (1, 2, 3%). The obtained microspheres were characterized for size, morphology, drug loading, ζ potential and swelling degree. Mucoadhesive properties were examined using texture analyzer and three different models of adhesive layers – gelatin discs, mucin gel and porcine vaginal mucosa. *In vitro* drug release, mathematical release profile and physical state of microspheres were also evaluated. The obtained results indicate that sodium alginate is a suitable polymer for developing mucoadhesive dosage forms of metronidazole. The optimal formulation F3 (drug : polymer ratio 1 : 2 and 1% alginate solution) was characterized by the highest metronidazole loading and sustained drug release. The results of this study indicate promising potential of ALG microspheres as alternative dosage forms for metronidazole delivery.

Keywords: metronidazole, sodium alginate, microspheres, spray drying technique, mucoadhesive properties

Multicompartment dosage forms, such as microparticles, provide high surface area of drug release and short diffusion way, which in the consequence enables improvement of therapeutic efficacy and reduction of drug toxicity (1). One of the advanced methods used in microparticles production is spray drying. Spray drying is a one-step process, which depends on spraying a solution, emulsion or suspension in a stream of drying gas - compressed air or nitrogen. Increased contact area between the phases leads to an intensive exchange of heat and almost immediate removal of the solvent to form dry particles. Parameters of the process, e.g., atomization devices, drying chambers, aspirator and feed rate, drying temperature, spray air flow and properties of the material affect the characteristics of the dried product. Properly selected parameters allow to obtain dry particles with desired properties (2).

One of the polymers used for preparation of microparticles is sodium alginate (ALG), which commonly occurs in seaweed. It is a heteropolysaccharide composed of monomers of β-D-mannuronic acid and α-L-guluronic acid. ALG possesses a number of advantages: non-toxicity, biocompatibility and biodegradability. In addition, mucoadhesive properties of this polymer can increase the residence time of the dosage form and in the consequence improve the drug bioavailability (3–5).

Metronidazole (MT) is synthetic, chemotherapeutic drug, which is a derivative of nitroimidazole. Its activity includes strictly anaerobic bacteria and protozoa. Mechanism of MT action involves the penetration of bacterial cells in the process of passive diffusion and generation of active product in the reduction of the nitro group, which causes damage of cellular DNA (6, 7). MT is one of the most effective drugs in the eradication of *Helicobacter pylori*, which resides mainly in the gastric mucosa and is an etiologic factor in the development of the gastritis, gastric ulcer and gastric carcinoma (8). MT is also widely used to treat trichomoniasis – the most common nonviral sexually transmitted disease, which affects about 170 million people a year and is caused by the protozoan *Trichomonas vaginalis* (9). Registered preparations with MT include tablets for oral or vaginal administration, gels, creams and intravenous dosage forms. However, multicompartment sustained release forms with MT, which could provide greater uniformity and reproducibility of the delivered dose, are not available (10), therefore the

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objective of this study was to prepare MT loaded ALG mucoadhesive microspheres by the spray drying technique. The effect of the drug : polymer ratio and concentration of ALG solution on the properties of prepared microspheres was evaluated. The obtained microspheres (formulations F1-F9) were characterized for size, morphology, drug loading, entrapment efficiency, swelling properties and ζ potential. Mucoadhesive properties of the microspheres were examined by using texture analyzer and three different models of adhesive layer: gelatin discs, mucin gel and porcine vaginal mucosa. The physical state of microspheres was determined by differential scanning calorimetry. The *in vitro* drug release and mathematical modeling of MT release were also evaluated.

EXPERIMENTAL

Materials

Metronidazole (MT) was purchased from Amara (Kraków, Poland). Sodium alginate (ALG) low viscosity (a viscosity of 2% solution: 100-300 cP) was purchased from Sigma Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid, sodium chloride, potassium hydroxide, calcium hydroxide, lactic acid, acetic acid, glycerol, urea and glucose were obtained from Chempur (Piekary Śląskie, Poland). Water was distilled and passed through a reverse osmosis system Milli-Q Reagent Water System (Billerica, MA, USA). Mucin type II from porcine stomach and gelatin type B from bovine skin was purchased from Sigma Aldrich (Steinheim, Germany). Porcine vaginal mucosa from large white pigs weighting ≈ 200 kg was obtained from the veterinary service (Turośń Kościelna, Poland). Samples were stored at -20°C and before the experiment were defrosted and cut into 5 mm in diameter and 2 mm thick pieces.

Preparation of microspheres

Microspheres were produced using Büchi Mini Spray Dryer B-290 (Büchi, Flawil, Switzerland). In order to choose the optimal parameters of spray drying to obtain product of the desired properties, a number of tests were conducted and the experimental parameters of the process were set as follows: inlet temperature 150°C, aspirator flow 37 m^3/h, feed flow 5 mL/min, spray flow 600 L/h. Microspheres (F1–F9) were prepared by using different drug : polymer ratio (1 : 1, 1 : 2, 2 : 1) and different concentrations of sodium alginate (1, 2, 3%) (Table 1).

Evaluation of microspheres

Morphology and particle size distribution

Measurements of the particle size and mean diameter of microspheres were performed using an optical microscope equipped with a camera (Motic BA400, Wetzlar, Germany) and Zetasizer NanoZS90 (Malvern Instruments, Malvern, United Kingdom). Morphology of the microspheres formulation F3 (with the highest MT loading) was additionally examined by scanning electron microscope (SEM) (Hitachi S4200, Tokyo, Japan).

Table 1. Characteristics of MT loaded ALG microspheres (formulation F1–F9).

Formulation	Drug: polymer ratio	ζ potential (mV)	Production yield (%)	Encapsulation efficiency (%)	Percent loading (%)	Mean diameter (μm)
	1% ALG solution					
F1	1:2	- 61.33 ± 9.71	51.15 ± 1.81	92.80 ± 2.32	35.88 ± 1.16	0.74 ± 0.04
F2	1:1	- 66.80 ± 9.53	46.92 ± 1.25	94.52 ± 2.13	51.19 ± 1.38	1.51 ± 0.13
F3	2:1	- 71.93 ± 7.92	42.49 ± 1.42	91.07 ± 1.91	62.17 ± 1.89	1.91 ± 0.15
2% ALG solution						
F4	1:2	- 65.60 ± 9.74	44.69 ± 1.22	76.28 ± 2.96	34.81 ± 1.34	2.27 ± 0.13
F5	1:1	- 66.80 ± 8.32	40.25 ± 1.76	86.36 ± 2.65	39.52 ± 2.48	2.24 ± 0.14
F6	2:1	- 72.77 ± 9.21	31.04 ± 1.88	88.31 ± 3.19	51.43 ± 1.71	1.29 ± 0.19
3% ALG solution						
F7	1:2	- 67.77 ± 8.91	35.59 ± 1.17	85.69 ± 3.29	34.94 ± 1.51	2.95 ± 0.15
F8	1:1	- 66.87 ± 8.46	38.18 ± 1.36	89.40 ± 2.74	44.86 ± 2.26	3.96 ± 0.16
F9	2:1	-70.57 ± 8.02	30.68 ± 1.62	79.31 ± 2.15	47.70 ± 2.44	1.84 ± 0.13

HPLC analysis of MT

The concentration of MT was determined by the HPLC system Agilent Technologies 1200 equipped with a G1312A binary pump, a G1316A thermostat, a G1379B degasser and a G1315B diode array detector (Agilent, Waldbronn, Germany). Data collection and analysis were performed using Chemstation 6.0 software. Isocratic separation was achieved on a Zorbax Eclipse XDB–C18, 4.6 × 150 mm, 5 µm column (Agilent, Waldbronn, Germany). Mobile phase was acetonitrile : 0.01 M phosphate buffer pH 4.7 (15 : 85, v/v), the flow rate was 1.0 mL/min and UV detection was performed at a wavelength of 319 nm (11). The column temperature was maintained at 25°C. For injection into the HPLC system, 20 µL of sample was used. All reagents used for analysis were HPLC grade. The retention time of MT was 3.0 min and retention factor (k) was 2. Retention factor (k) was determined by using the formula: $k = t_R - t_0 / t_0$, where t_R – retention time of the analyte on the column, t_0 – retention time of a non-retained compound (12). Standard calibration curve was linear over the range of 1–100 µg/mL with the correlation coefficient (R^2) 0.999.

Determination of MT loading, encapsulation efficiency and production yield

MT loading in the microspheres was determined by dissolving an accurately weighted amount of microspheres (20 mg) in 10 mL of distilled water and agitating for 24 h at 150 rpm in a water bath (13). After filtration through 0.45 µm cellulose acetate (CA) Millipore filters (Billerica, MA, USA), concentration of MT was determined by HPLC method. Each sample was analyzed in triplicate. The mean drug encapsulation efficiency was calculated by formula:

$$EE = Q_a / Q_t \times 100$$

where EE – percentage of encapsulation efficiency, Q_a – actual drug content, Q_t – theoretical drug content.

The percentage yield of MT in the ALG microspheres was determined by using the formula:

$$Y = W_m / W_t \times 100$$

where Y – percentage production yield, W_m – weight of microspheres and W_t – theoretical weight of drug and polymer (13).

ζ potential

ζ potential measurements were performed using a Zetasizer NanoZS90 (Malvern Instruments, Malvern, United Kingdom).

Swelling properties

Swelling properties of microspheres were examined in modified simultaneous vaginal fluid (SVF) pH 4.2 or in 0.1 M HCl pH 1.2. SVF was prepared by dissolving sodium hydroxide, hydrochloric acid, sodium chloride, potassium hydroxide, calcium hydroxide, lactic acid, acetic acid, glycerol, glucose and urea in water and adjusted to pH 4.2 by using 0.1 M HCl (14). Microspheres (20 mg) were placed in beakers containing 25 mL of medium and stirred at 100 rpm at 37 ± 1°C. The microspheres were periodically weighted at predetermined time interval until a constant weight was obtained. The swelling ratio (SR) was calculated by using the following formula:

$$SR = (Ws - Wo)/Wo$$

where Wo – initial weight of microspheres and Ws – weight of microspheres after swelling (15).

Mucoadhesive properties

Evaluation of mucoadhesiveness was performed using TA.XT.Plus Texture Analyser (Stable Micro Systems, Godalming, United Kingdom) and three different models of adhesive layers: gelatin discs, mucin gel and porcine vaginal mucosa. Experimental parameters of the process were chosen during preliminary tests and set as follows: pretest speed 0.5 mm/s, test speed 0.1 m/s, contact time 180 s, post test 0.1 mm/s, applied force 1 N. Gelatin discs were prepared by pouring 30% w/w aqueous solution into a Petri dish. Layer of mucin was prepared by absorbing of 10% mucin gel on a discs with cellulose fiber (5 mm in diameter). Tests were conducted at 37 ± 1°C. Each adhesive layer was adhered to an upper probe and moisturized with SVF. Mucoadhesive properties were determined as maximum detachment force (F_{max}) and work of mucoadhesion (W_{ad}) – calculated from the area under the force *versus* distance curve and expressed in µJ.

In vitro MT release

MT release profiles were obtained according to the modified USP method using dissolution basket apparatus (Erweka Paddle Dissolution tester type DT 600HH, Heusenstamm, Germany). The receptor compartment was filled with 500 mL of modified simulated vaginal fluid (SVF) (pH 4.2) or 0.1 M HCl (pH 1.2) to provide *sink* conditions. All formulations of microspheres (containing 250 mg of MT for study in 0.1 M HCl or 500 mg of MT for study in SVF) were suspended in dissolution medium and stirred at 50 rpm at 37 ± 1°C for 8 h. Samples were

withdrawn and filtered through 0.45 μm CA Millipore filters (Billerica, MA, USA) at predetermined time intervals and replaced with fresh dissolution medium (16). The amount of released MT was analyzed by HPLC method (as described earlier). The studies were carried out in triplicate.

Mathematical modeling of MT release profile

MT release data were analyzed according to zero order kinetic, first order kinetic, Higuchi model and Korsmeyer–Peppas equation to characterize mechanism of the drug release. Constants of release kinetics and regression coefficients (R^2) were calculated from the slope of plots by linear regression analysis.

Zero order kinetic describes formula:

$$Q_t = Q_o + K_o t$$

where Q_t is the amount of drug dissolved in time t, Q_o – the initial amount of drug in the solution, and K_o – the zero order release constant.

First order kinetic formula:

$$log\ Q_t = log\ Q_o + kt/2.303$$

where Q_t is the amount of drug released in time t, Q_o – the initial amount of the drug in the solution and k – the first order release constant.

Higuchi model describes equation:

$$Q = kHt^{1/2}$$

where Q is cumulative amounts of the drug released at time t and kH – the Higuchi dissolution constant.

Korsmeyer-Peppas model is expressed by the following equation:

$$M_t / M_8 = kt^n$$

where M_t and M_8 are amounts of the drug released at time t and infinite time, k is the constant incorporating structural and geometrical characteristics and n – diffusion release exponent used to interpretation of diffusion release mechanism (17, 18).

Differential scanning calorimetric studies

Differential scanning calorimetric (DSC) analysis of MT, ALG and formulation F3 of microspheres (with the highest drug loading) was performed using an automatic thermal analyzer system (DSC TEQ2000, TA Instruments, New Castle, DE, USA). Each sample was precisely weighted (5 mg) and placed in sealed aluminium pans. An empty pan was used as a reference. Temperature calibrations were performed using indium and zinc as standard. Samples were heated from 25 to 230°C at scanning rate of 10°C/min under nitrogen flow of 20 mL/min (19).

Statistical analysis

Quantity variables were expressed as the mean and standard deviation. Statistical analysis was performed using nonparametric Kruskal-Wallis test and conducted by using STATISTICA 10.0 software. Differences between groups were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Frequently encountered problems with conventional dosage forms are differences in bioavailability, relatively short residence time in the gastrointestinal tract and possibility of irritation caused by local drug release (20, 21). In contrast, multicompartment dosage forms due to the high surface area offer numerous advantages, which include reduction of individual differences in bioavailability, improved efficacy and reduced toxicity (22). Drug absorption in the stomach is a complex process which depends on physiological factors: pH, presence of food, peristalsis and stomach emptying. In acidic fluid, the sodium alginate is converted to the insoluble, swelling alginic acid. Application of ALG to obtain mucoadhesive microspheres with MT could prolong residence time in the stomach and improve effectiveness in the eradication of *H. pylori* (23, 24). MT is also one of the most effective drugs for common vaginal infections – bacterial vaginosis and trichomoniasis. However, the currently available traditional vaginal delivery systems with MT have limitations. ALG, as anionic polymer with mucoadhesive properties, could prolong residence time of the dosage form in the site of application and in the consequence improve therapeutic efficacy of MT (25, 26).

Characteristics of ALG microspheres with MT

In the present study, MT loaded microspheres were successfully prepared by the spray drying method using sodium alginate as polymer matrix. Spray drying technique is relatively simple to carry out, however obtaining the product with desired properties is a complex process, which depends on several factors. The optimal spray drying process parameters included: inlet temperature 150°C, aspirator flow 37 m^3/h, feed flow 5 mL/min, spray flow 600 L/h. The characteristics of microspheres obtained using different drug : polymer ratio and different concentration of ALG solution are shown in Table 1. The mean diameter of microspheres ranged from 0.74 ± 0.04 μm (F1) to 3.96 ± 0.16 μm (F8). The minimum drug loading was observed in formulations F1, F4, F7, when drug : polymer ratio was 1 : 2. Maximum drug loading was in formulation F3 (drug : polymer ratio 2 : 1, 1% ALG solution). The encapsulation efficiency ranges from

76.28 ± 2.96% (F4) to 94.52 ± 2.13% (F2) (Table 1). The increase in the drug : polymer ratio resulted in an improvement of MT percent loading, but entrapment efficiency was slightly decreased, which could be caused by the insufficient amount of ALG to cover MT particles completely. The results obtained from tests performed with an optical microscope indicated that all formulations of microspheres had spherical shape, smooth surface and did not aggregate (Fig. 1). The morphology of microspheres formulation F3 (with the highest MT loading) examined by scanning electron microscopy (SEM) is shown in Figure 2.

ζ potential

The stability of many systems is directly related to the magnitude of their ζ potential. In general, if the value of this parameter is more negative or more positive, the system is more stable. Conversely, if the microspheres ζ potential is relatively closer to zero, the particles have a tendency to aggregate. All performed formulations of microspheres possessed negative ζ potential with value from -61.33 ± 9.71 to -72.77 ± 9.21 mV (Table 1). The negative charge of the microparticle surface is mainly a result of the negative charge of ALG and -CH_2-CH_2-OH group of MT (27). ALG is anionic polymer with carboxyl and sulfate functional groups, which give rise to an overall negative charge at pH values exceeding the pKa of the polymer. It was also observed that value of ζ potential decreased with an increased amount of loaded MT.

Swelling and mucoadhesive properties

Swelling is the first step in the formation of bonds between polymer and mucous membrane and

Table 2. Mucoadhesive properties of formulations F1-F9.

Formulation	Type of adhesive layer					
	Gelatin		Mucin		Porcine vaginal mucosa	
	F_{max} [N]	W_{ad} [μJ]	F_{max} [N]	W_{ad} [μJ]	F_{max} [N]	W_{ad} [μJ]
F1	0.12 ± 0.91	79.77 ± 0.17	0.22 ± 1.15	113.30 ± 0.41	0.19 ± 0.96	134.90 ± 0.36
F2	0.15 ± 1.10	89.23 ± 0.15	0.18 ± 0.98	112.50 ± 0.23	0.20 ± 1.15	136.43 ± 0.28
F3	0.23 ± 0.90	119.87 ± 0.45	0.27 ± 1.51	163.70 ± 0.52	0.15 ± 1.98	141.57 ± 0.25
F4	0.12 ± 0.91	86.00 ± 0.15	0.22 ± 1.85	104.60 ± 0.24	0.20 ± 1.91	175.30 ± 0.19
F5	0.18 ± 0.82	152.00 ± 0.26	0.31 ± 0.89	158.30 ± 0.82	0.23 ± 1.34	207.37 ± 0.65
F6	0.21 ± 0.58	153.80 ± 0.45	0.32 ± 1.67	183.83 ± 0.26	0.24 ± 0.67	262.27 ± 0.88
F7	0.11 ± 1.71	62.93 ± 0.14	0.11 ± 1.90	140.30 ± 0.24	0.14 ± 1.92	145.57 ± 0.19
F8	0.22 ± 1.23	100.60 ± 0.52	0.21 ± 1.56	138.30 ± 0.77	0.25 ± 1.35	160.23 ± 0.31
F9	0.24 ± 0.98	136.50 ± 0.49	0.19 ± 0.89	148.90 ± 1.10	0.28 ± 0.59	179.27 ± 0.28

F_{max} – Maximum detachment force. W_{ad} – Work of adhesion

Table 3. Models of MT release from formulations F3, F6 and F9 in SVF or 0.1 M HCl.

Formulation	Zero order kinetics		First order kinetics		Higuchi model		Korsmeyer-Peppas model		
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	K_{KP}	n
SVF (pH 4.2)									
F3	0.354	7.188	0.973	0.323	0.748	10.00	0.976	0.424	0.191
F6	0.564	8.969	0.995	0.260	0.881	20.82	0.956	0.412	0.205
F9	0.477	8.408	0.959	0.293	0.791	17.37	0.909	0.404	0.184
0.1 M HCl (pH 1.2)									
F3	0.805	12.75	0.918	0.372	0.936	40.07	0.956	0.675	0.525
F6	0.771	12.14	0.957	0.353	0.925	36.89	0.954	0.633	0.463
F9	0.792	11.85	0.982	0.281	0.927	37.01	0.955	0.662	0.511

in the creating a spatial network with adhesive properties (28). As it is shown in Figure 3A, in microspheres examined in modified SVF, an initial rapid rise of swelling ratio (SR) in the first 30 min in all formulations was observed. The maximum swelling was observed in formulation F4. It was also noted that MT : ALG ratio had significant effect on SR. Formulations with higher MT loading and lower ALG content (F3, F6 and F9) were characterized by lower swelling ability (Fig. 3A). Due to the faster conversion of sodium alginate to the insoluble alginic acid in more acidic pH, the rise of SR in microspheres evaluated in 0.1 M HCl was significantly weaker (Fig. 3B).

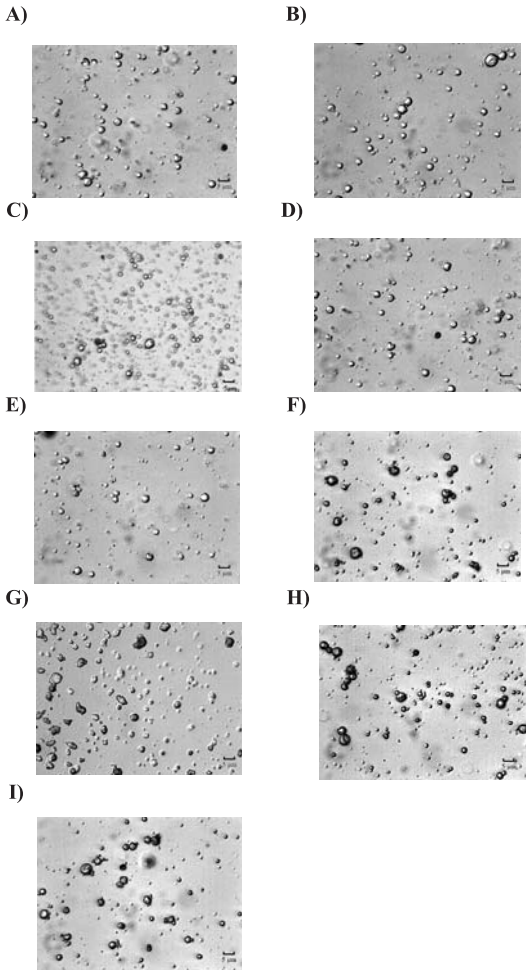

Figure 1. Microscopic images of microspheres: F1 (**A**), F2 (**B**), F3 (**C**), F4 (**D**), F5 (**E**), F6 (**F**), F7 (**G**), F8 (**H**) and F9 (**I**) under magnification 100×

Mucoadhesive properties were investigated using TA.XT.Plus Texture Analyser and gelatin discs, mucin gel and porcine vaginal mucosa as different adhesive layers. Porcine vaginal mucosa model is often used to reflect behavior of dosage forms *in vivo* because of its similarity to human mucosa in terms of histology, ultrastructure and composition (29). It was found that all formulations were characterized by mucoadhesive properties (Table 2). ALG swells and facilitates the adhesive interactions with mucosa and contributes to the formation of a cohesive layer. The numerous carboxyl functional groups of ALG can form hydrogen bonds with mucin molecules, thus producing some adhesive force and electrostatic interactions between polymer and adhesive layer (30, 31). Unexpectedly, higher work of adhesion was observed in formulations with lower amount of ALG (F3, F6 and F9).

In vitro MT release and mathematical models of release profile

In vitro release of MT was examined from formulations F3, F6, F9 (with the highest MT loading) in SVF (pH 4.2) or in 0.1 M HCl (pH 1.2) (Fig. 4). In SVF, after 0.5 h of the study, significant burst release of MT was observed (75.76 ± 3.76%, 55.32 ± 4.17% and 61.26 ± 3.91% of MT was released

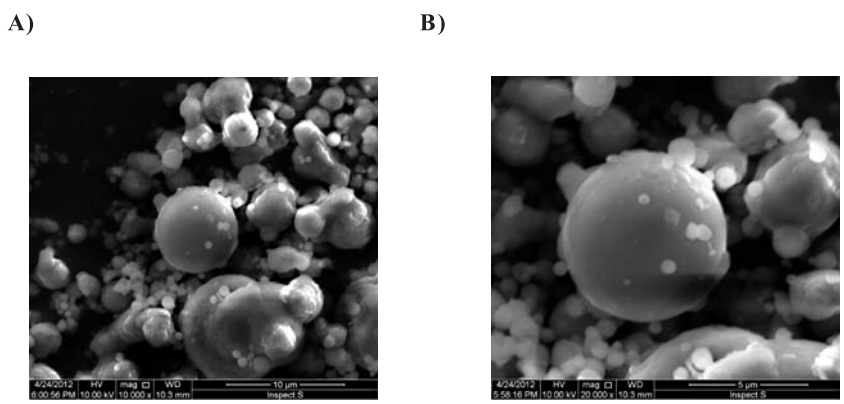

Figure 2. SEM images of microspheres formulation F3 under magnification 10 000× (**A**), 20000× (**B**)

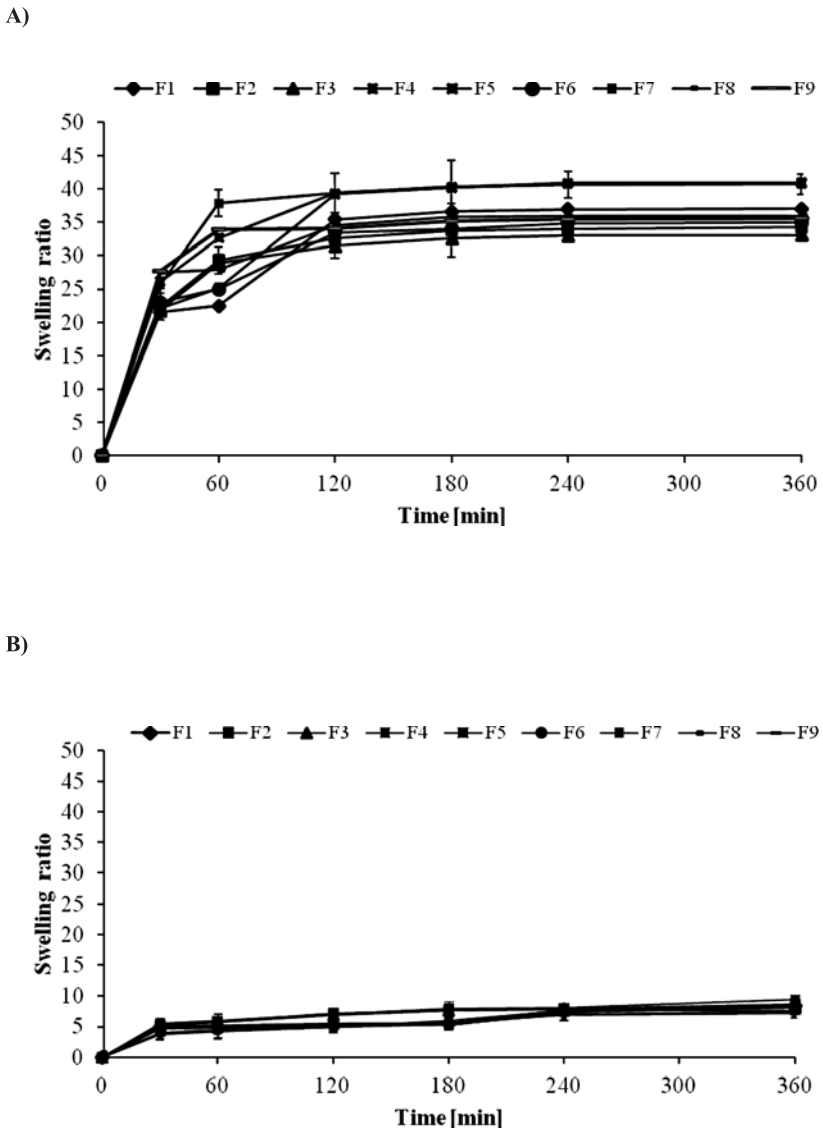

Figure 3. Swelling ratio (SR) of microspheres formulations F1-F9 in SVF (pH 4.2) (**A**) and in 0.1 M HCl (pH 1.2) (**B**)

from F3, F6 and F9, respectively) and drug was continuously released up to 4 h (Fig. 4A). Contrary, in 0.1 M HCl there was no burst effect, MT release was significantly slower (80% of MT was released after 3 h) and sustained up to 6 h (Fig. 4B). Sustained MT release in 0.1 M HCl is caused by the higher (at acidic pH) conversion of sodium alginate to the insoluble alginic acid.

Mechanism of MT release from obtained microspheres was analyzed according to various mathematical models: zero order kinetic, first order kinetic, Higuchi model and Korsmeyer–Peppas equation to find out the coefficient of correlation (R^2) and *n* value (Table 3). In Korsmeyer-Peppas model, *n* is the release exponent and provides information about mechanism of the drug release from different geometry. When *n* takes a value of 0.43, it indicates diffusion mechanism, $n = 0.85$ - indicates swelling-controlled release, *n* between 0.43 and 0.85 – mechanism of drug release, which includes both phenomena (anomalous transport) (18). In all formulations examined in SVF, the obtained *n* values were below 0.43 indicating diffusion as MT release mechanism. Interestingly, in 0.1 M HCl solution, value of *n* was above 0.43 indicating anomalous transport based on both diffusion and swelling con-

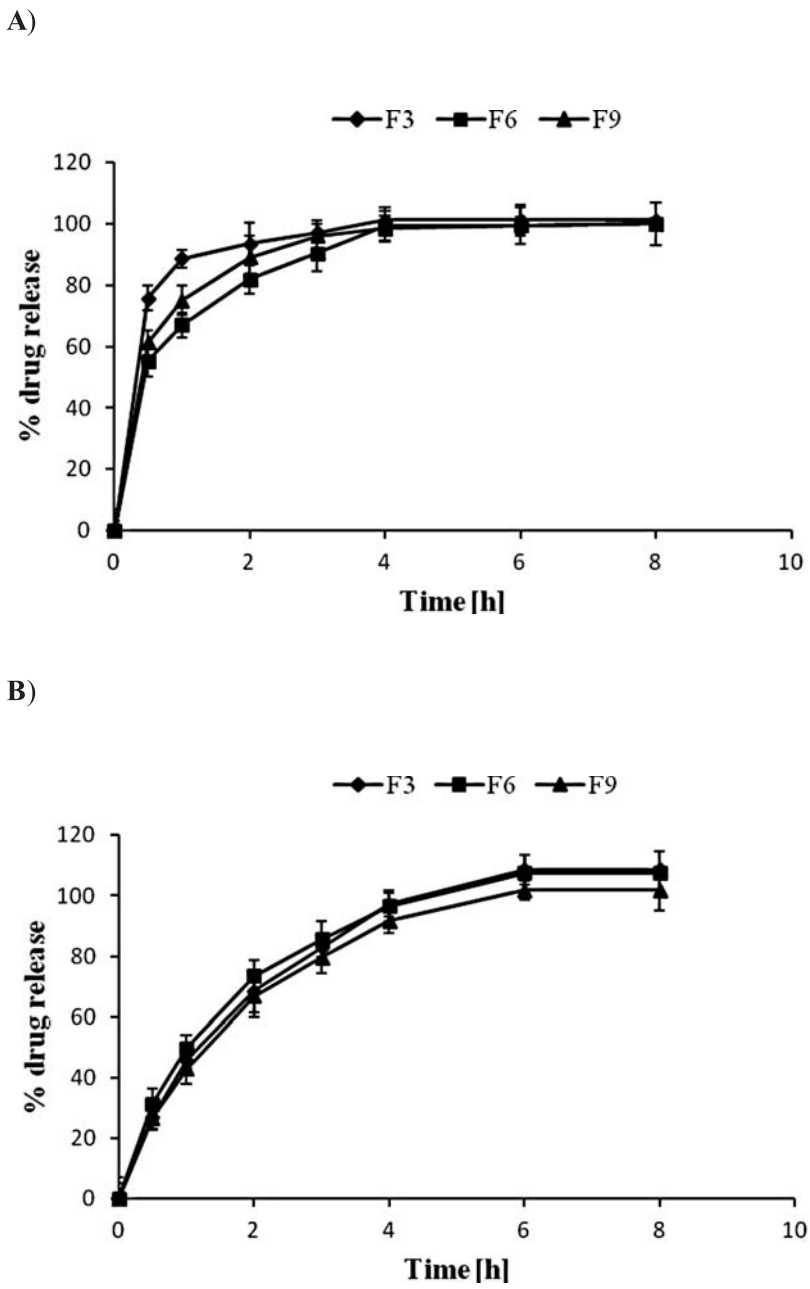

Figure 4. MT release from formulations F3, F6 and F9 in SVF (pH 4.2) (**A**) and 0.1 M HCl (pH 1.2) (**B**

trolled release. In all formulations, the highest value of R^2 was observed in first order kinetic model, which indicates that MT release was concentration dependent (Table 3).

Differential scanning calorimetric studies

The physical state of MT inside ALG microspheres was assessed by thermal analysis. DSC thermograms of MT, ALG and microspheres of formulation F3 (with the highest drug loading) are shown in Figure 5. Under the experimental conditions, endothermic peak of sodium alginate close to 108.25°C was found indicating glass transition of the polymer, and MT exhibited a sharp endothermic peak at 163.45°C, corresponding to the melting point of pure MT. In thermogram of microspheres F3 sharp peak of MT was observed, suggesting that drug was stable within the polymer matrix (32).

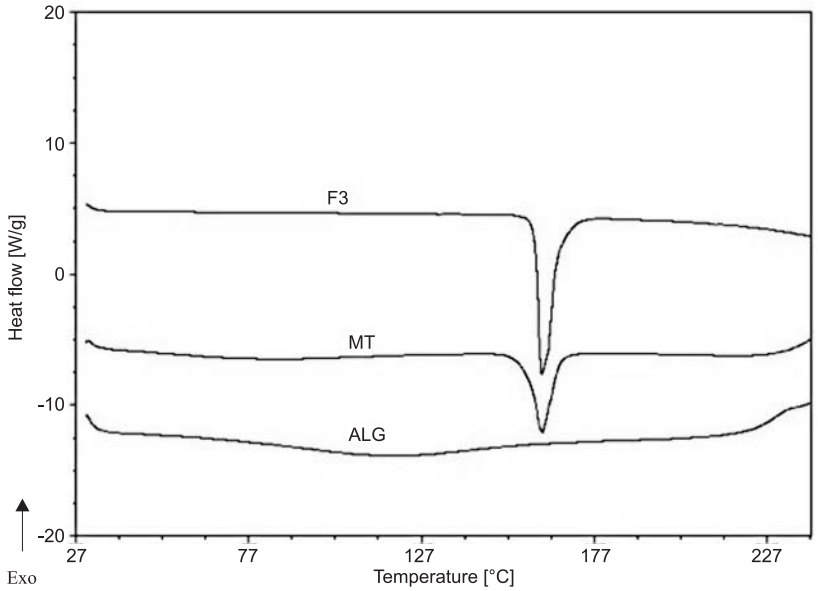

Figure 5. DSC thermograms of MT, ALG and microspheres of formulation F3

CONCLUSIONS

The obtained data suggest that ALG microspheres with MT can be successfully prepared by the spray drying technique. MT release and mucoadhesive properties of the microspheres can be altered by varying the drug : polymer ratio and concentration of polymer. The optimal formulation of microspheres – F3 is characterized by the highest MT loading and sustained MT release (up to 4 h in SVF and up to 6 h in 0.1 M HCl). All microspheres possess swelling and mucoadhesive properties. The results of this study indicate promising potential of ALG microspheres as alternative dosage forms for MT delivery. Preparation and evaluation of gelatin capsules containing ALG microspheres with MT and tableting these microspheres is underway and will be described in a due course.

Acknowledgments

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PHARMACOLOGY

THE EFFECT OF SEROTONIN 5-HT$_{1A}$, 5-HT$_2$ RECEPTOR LIGANDS, KETOPROFEN AND THEIR COMBINATION IN MODELS OF INDUCED PAIN IN MICE

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Anstract: The present study was carried out to investigate the effects of the 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione (compounds **1**-**4**) in two animal models of induced pain and to compare their effects with ketoprofen and with their combination. All experiments were performed on albino mice. Mice were evaluated for their responsiveness to noxious stimuli using: the hot-plate test and the phenylbenzoquinone- induced writhing test. All compounds showed analgesic activity only in the writhing test. The analgesic activities of compounds **3** and **4** were similar to ketoprofen. The compounds slightly increased the analgesic effect of ketoprofen when used in combination in the visceral type of pain. The possible mechanisms of the antinociceptive effect of these compounds are thought to involve the activation of analgesic effect mediated by the serotonergic pathways or combination of this mechanism with other important mediators playing a role in pain modulation.

Keywords: analgesic activity, serotonergic receptors, writhing test, xanthine derivatives

Chronic pain represents one of the most important problems. This is a common unpleasant sensory and emotional experience associated with actual or potential tissue damage. For instance, it has been estimated that 10-13% of the general population in Europe, are being disabled by persistent pain. Conventionally, analgesic treatment is accomplished by three principal drug groups: nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and local anesthetics, administered through different routes and modalities (1). Pain relief can be achieved and adverse effects minimized by a combination of analgesics (2). Analgesics are complemented with co-analgesic (adjuvant) drugs, specially in pain of inflammatory and neuropathic type (1, 3, 4). In the case of neuropathic pain, treatment with opioids may be of limited efficacy and combination with co-analgesics is necessary (4–8).

Co-analgesic drugs are defined as drugs that have a primary indications other than pain but are analgesic in some painful conditions or are capable of decreasing the side effects of analgesic drugs (4). Co-analgesics can also be defined as drugs that have weak or non-existent analgesic action when administered alone, but can enhance analgesic actions when co-administered with known analgesic agents (4). Such combinations increase analgesia without increasing the dose of analgesics, and therefore, can reduce the incidence of adverse effects (4). The combined treatment with the two types of drugs at doses much lower than therapeutic doses may be of great value in pain therapy (9).

Co-analgesic drugs include antidepressants, antipsychotics, anticonvulsants, antiarrhythmics, corticosteroids and others (4, 8, 10). Efficacy of these drugs depends on the type of pain, its pathophysiology, clinical status and adequacy of pain intervention. There has been an increase in the number of these drugs and they now play an important role in the management of chronic pain (10).

In chronic pain, antidepressants are an essential part of the therapeutic strategy in addition to classical analgesics (3, 8, 10). These drugs belong to the groups of tetracyclic antidepressants (TeCAs) (amoxapine, maprotiline), tricyclic antidepressants (TCAs) (amitriptyline, doxepin, imipramine), selec-

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tive serotonin reuptake inhibitors (SSRIs), serotonin and noradrenaline reuptake inhibitors (SNRIs) (duloxetine, venlafaxine, milnacipran), and atypical antidepressants (bupropion, trazodone, mirtazapine, nefazodone), (3, 8). Antidepressants effective in chronic pain act *via* different mechanisms as can be seen in Table 1, (3, 10). However, other mechanisms have also been suggested..

Antidepressants are commonly used to treat the following chronic pain conditions: arthritis, central pain syndrome, fibromyalgia, low back pain, migraines, nerve damage from diabetes (diabetic neuropathy) (8, 11). Their effectiveness is best documented for painful diabetic neuropathy. On the other hand, some clinical data suggest that headache can occur after prolonged use of amitriptyline in patients with depression but without pain symptoms (3). The explanation of this phenomenon requires further research.

The analgesic potency of antidepressant drugs has been suggested to result from the inhibition of monoamine reuptake in the central nervous system, which consequentially leads to an increased activity of the antinociceptive descending pathways (3). The antidepressants have an analgesic effect that may be, at least partly, independent of their effect on depression (3). The dose necessary to achieve optimal analgesia is usually lower than that required for antidepressant therapy, which may suggest separation of analgesic and antidepressant effect (3).

The present study is, therefore, intended to investigate the analgesic potential of 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione, used alone or in combination with ketoprofen in two models of induced pain in mice, representing different types of pain stimuli. Our previous study demonstrated a potent antidepressant-like activity of several derivatives of 8-alkoxypurine-2,6-dione in the forced swimming test in mice and showed their strong affinity for the 5-HT_2 and 5-HT_{1A} receptors (12).

EXPERIMENTAL

Chemistry

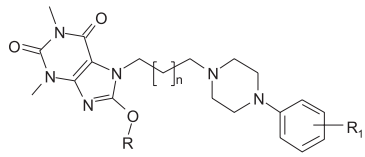
The structures of the investigated compounds **1**–**4** are presented in Table 2. Compounds **1**–**4** were synthesized by nucleophilic substitution of previously obtained 7-chloroalkyl-8-alkoxy-1,3-dimethyl-3,7-dihydropurine-2,6-diones with the appropriate phenylpiperazines in the presence of K_2CO_3. The synthesis and physicochemical data of compounds **1**–**4** were described elsewhere (13). The investigated compounds were pharmacologically tested as hydrochloride salts.

Table 1. The mechanism of action of antidepressants effective for chronic pain (3, 11).

Group	Drugs	**Mechanisms**
TCA	Amitriptyline Doxepin Imipramine	5-HT > NE reuptake inhibition
TeCA	Amoxapine	NE and 5-HT reuptake inhibition
SSRI	Citalopram Fluoxetine Paroxetine Sertraline Fluvoxamine	5-HT > NE reuptake inhibition
SNRI	Duloxetine Venlafaxine Milnacipran	5-HT NE > DA reuptake inhibition
Atypical antidepressant	Bupropion Trazodone Mirtazapine Nefazodone	DA NE reuptake inhibition 5-HT_2 receptor blockade 5-HT reuptake inhibition a_2-NE and 5-HT_2 presynaptic agonist; 5-$HT_{2/3}$ receptor blockade 5-HT_2 receptor blockade 5-HT reuptake inhibition

TCA - tricyclic antidepressants, TeCA - tetracyclic antidepressants, SSRI - selective serotonin reuptake inhibitors, SNRI - serotonin and noradrenaline reuptake inhibitors; NE - noradrenaline, DA - dopamine, HT - hydroxytryptamine.

Table 2. Structure and binding affinity data for serotonin 5-HT_{1A} and 5-HT_2 receptors of the investigated compounds.



Compound	R	n	R_1	K_i (nM) ± SEM	
				5-HT_{1A}	5-HT_2
1	C_3H_7	2	m-Cl	15 ± 1[a]	28 ± 2[a]
2	C_3H_5	2	m-Cl	12 ± 2[a]	15 ± 1[a]
3	C_2H_5	1	m-Cl	190 ± 28[a]	23 ± 2[a]
4	C_3H_7	1	m-Cl	288 ± 18[a]	25 ± 2[a]

[a] Data taken from ref. (12).

Table 3. The influence of the investigated compounds and ketoprofen on the pain reaction in the "writhing syndrome" test in mice.

Compound	Dose mg/kg	Mean number of writhings ± SEM
Control		30.5 ± 0.7
1	5	8.7 ± 0.8**
2	5	9.3 ± 0.2**
3	5	4.7 ± 0.6***
4	5	3.9 ± 0.4***
Ketoprofen	5	1.9 ± 0.1***

Data are presented as the means ± SEM of 6-8 mice per group. The results were analyzed by Student's *t*-test. ** $p < 0.01$,*** $p < 0.001$ *vs.* control.

Animals

The experiments were carried out on male albino-Swiss mice (body weight 20-26 g). The animals were housed in constant temperature facilities exposed to 12 : 12 h light-dark cycle and maintained on a standard pellet diet, tap water was *ad libitum*. Control and experimental groups consisted of six to eight animals each. The investigated compounds were administered intraperitoneally (*i.p.*) as a suspension in 0.9% NaCl.

Statistical analysis

The statistical significance was calculated using Student's *t*-test. Differences were considered statistically significant at $p \leq 0.05$.

Methods

The writhing syndrome test

Mice were treated with 0.25 mL of 0.02% phenylbenzoquinone solution 30 min after *i.p.* administration of the investigated compound or the vehicle. Then, the mice were placed individually in glass beakers and 5 min were allowed to elapse. After that period, each animal was observed for 10 min and the number of characteristic writhes was counted. The control group was given *i.p.* 0.9% NaCl. The analgesic effect of the tested substances was determined by a decrease in the number of writhes observed (14). The ED_{50} values and their confidence limits were estimated by the method of Litchfield and Wilcoxon (15).

The hot plate test

In the hot plate test, mice were treated *i.p.* either with the test compound or the vehicle 30 min before placing the animal on a hot plate apparatus (Hot Plate 2A Type Omega) with the temperature controlled at 55-56^{o}C. The time elapsing until the animal licks its hind paws or jumps is recorded using a stop-watch (16). The ED_{50} values and their

confidence limits were estimated by the method of Litchfield and Wilcoxon (15).

RESULTS

Analgesic activity in the writhing syndrome test

All xanthine derivatives were tested in the writhing test. In this test, pain was induced by injection of an irritant (such as phenylbenzoquinone) into the peritoneal cavity of mice. In this test, the investigated compounds (**1**–**4**) administered at a dose 5 mg/kg, showed significant analgesic properties (Table 3). Ketoprofen was a drug of reference in this assay. The mean number of writhing responses in the vehicle-treated mice was 30.5. The strongest analgesic effect was observed for compounds **3** and **4**. Compound **3** injected *i.p.* at a dose 5 mg/kg body weight, reduced the number of writhes in response to an irritating stimulus by 84.6%. Administration of compound **4** at a same dose, produced a strong analgesic effect (inhibition by 87.2%). Compound **1** reduced the number of abdominal writhings by 71.4% with respect to the control group, while compound **2** statistically significantly diminished the number of abdominal constrictions by 69.5%, as compared to the 0.9% NaCl-pretreated animals.

In this model of pain, each drug was used alone to test its analgesic effect, and in combination with ketoprofen (at the dose 5 mg/kg) to look for a possible potentiation of its analgesic effect.

The combination of the investigated compound **3** and ketoprofen produced a slightly higher effect in the writhing test, compared to each of them used alone (reduction of the number of writhes by 96.8%), (Table 4). The combination of the investigated compound **4** and ketoprofen also produced a slightly higher effect in the writhing test, compared to each of them used alone (reduction by 97.6%). As regards compound **2** and ketoprofen, this combination also increased the analgesic activity in the writhing test (95.5% increase *versus* 69.5 and 93.7%, respectively), (Table 4). Compound **1** and ketoprofen reduced the number of writhes in response to an irritating stimulus by 95.2%. This combination produced higher effect in the writhing test, compared to compound **1** (71.4%) and ketoprofen (93.7) used alone.

Hot plate test

As demonstrated in Table 5, in the hot plate test the investigated compounds **1**-**4** did not exerted an analgesic activity at the dose of 5 mg/kg body weight. Morphine, which was a drug of reference in this assay, showed a high antinociceptive potency.

DISCUSSION AND CONCLUSION

Serotonin (5-hydroxytryptamine, 5-HT), like noradrenaline, is believed to be one of important modulators of painful stimulus transmission while spinal serotonergic receptors play an important role in the inhibition of nociceptive reaction connected with the release of stimulatory amino acids and substance P (17). Many types and subtypes of 5-HT receptors were identified in the CNS, however, only four of them - $5HT_{1-4}$ were found in the spinal cord (17). When administered intrathecally, 5-HT had an antinociceptive effect in acute pain models. In the periphery, 5-HT was shown to produce analgesic response in pain accompanying inflammatory process.

Animal studies demonstrated antinociceptive action of serotonin *via* spinal 5-HT receptors in the writhing test. Analgesic action of different substances in that test could result from their effect on the serotonergic system (18) probably mediated by $5\text{-}HT_2$ and $5\text{-}HT_3$ receptors. It was abolished by antagonists of these receptors (ketanserin, cyprohep-

Table 4. The influence of the combination of the investigated compounds and ketoprofen on pain reaction in the "writhing syndrome" test in mice.

Compound	Dose mg/kg	Mean number of writhings ± SEM
Control		31.5 ± 2.6***
1 + Ketoprofen	5	1.5 ± 0.8***
2 + Ketoprofen	5	1,4 ± 0.2***
3 + Ketoprofen	5	1.0 ± 0.1***
4 + Ketoprofen	5	0.8 ± 0.1***

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student's *t*-test. *** $p < 0.001$ *vs.* control.

Table 5. The influence of the investigated compounds and ketoprofen on the pain reaction in the hot plate test in mice.

Compound	Dose mg/kg	Time of reaction to ain stimulus (s) ± SEM
Control		30.0 ± 2.4
1	5	27.2 ± 1.3
2	5	29.2 ± 3.0
3	5	31.6 ± 2.2
4	5	28.3 ± 1.6
Morphine	5	50.2 ± 0.7 **
Ketoprofen	5	19.2 ± 3.1

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student's *t*-test. ** $p < 0.01$ *vs*. control.

tadine, ondansetron). The $5HT_1$ receptors seemed to play a less important role in this model of pain and their antagonists did not abolish nociceptive action of agonists. The role of $5HT_{1A}$ receptors in thermal pain models is unclear (17). It appears that $5HT_{1A}$ receptors do not play a role in the hot plate test and antinociceptive action in this test can result from the stimulation of serotonin type 3 and probably 2 receptors.

Summarizing, the problem of implication of specific serotonin receptor subtypes in different animal models of pain is very complex and deserves further clarification (19, 20). It should be remembered that the role of serotonin in the CNS and its receptor-mediated actions, including analgesic effect, are closely connected with other neurotransmitter systems (19, 20).

Animal studies revealed analgesic potential of antidepressant drugs which is partly mediated by serotonin receptors but can also stem from complex interactions between serotonergic, adrenergic and opioid systems (21). Due to their antinociceptive actions, some antidepressant drugs can be used as co-analgesics (3, 10, 11). Trazodone which presented a weak activity of a serotonin reuptake inhibitor and $5\text{-}HT_{2A}$ receptor antagonist, also showed analgesic activity in animal studies (22) but the clinical trials did not confirm its beneficial effects in chronic pain (3).

In our previous studies, all investigated compounds were demonstrated to be highly active $5\text{-}HT_{2A}$ receptor ligands. Compounds **3** and **4** showed 8.3- and 11.5-fold stronger affinity for $5\text{-}HT_2$ receptor than for $5\text{-}HT_{1A}$ whereas compounds **1** and **2** were found to be highly active $5\text{-}HT_{1A}$ receptor ligands. On the other hand, several derivatives of 8-alkoxypurine-2,6-dione revealed a potent antidepressant-like activity in the forced swimming test in mice (12). In our study, the most potent effect in the forced swimming test was produced by compounds **3** and **4** (12). The investigated compounds exhibited a similar mechanism of action as trazodone (23). Perhaps it was because of the combination of the weak activity of a serotonin reuptake inhibitor and a $5\text{-}HT_{2A}$ receptor antagonist.

Pain is often associated with inflammatory conditions. Non-steroidal anti-inflammatory drugs (NSAIDs) are the most commonly used analgesic agents. NSAIDs are clinically effective because they alleviate pain and inflammation (24-26) Thus, the NSAID (ketoprofen) was selected in the present study as a standard drug to be co-administered with the tested compounds (**1**–**4**), 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione.

The aim of the first stage of our studies was to determine analgesic activity of the test compounds and ketoprofen in the writhing test and the hot plate test.

7-(3-Chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione were administered at a dose of 5 mg/kg, because, as previous studies have shown, it was the lowest used dose that did not reduce the motility of animals (12). Higher doses of 10-30 mg/kg significantly decreased mobility. Therefore, this dose was used in order to exclude false-positive results in the tests assessing analgesic activity.

The analgesic activities of the 7-(3-chlorophenyl)piperazinylalkyl derivatives of 8-alkoxypurine-2,6-dione **1**–**4** were measured using the phenylbenzoquinone-induced writhing syn-

drome test. This test consists in intraperitoneal injection of the chemical irritant followed by subsequent counting of "writhes", i.e., characteristic contractions of abdominal muscles accompanied by a hind limb extensor motion. This test detects peripheral analgesic activity; however, some psychoactive agents (including clonidine and haloperidol) also show activity in this test. Compounds with anti-inflammatory properties, such as NSAIDs show a significant activity in this assay, viz. they abolish the reflex stimulated by the administration of an irritating substance, like phenylbenzoquinone

All tested compounds **1**–**4** demonstrated a statistically significant analgesic activity in this test with compounds **3** and **4** revealing the highest (similar to ketoprofen) potency. However, the observed effect was not stronger than that of ketoprofen alone at the same dose. It is probable that analgesic activity of the test compounds is mediated by 5-HT_2 receptors because they bound several times more strongly to 5-HT_2 than to 5-HT_{1A} receptors. It was additionally confirmed by the fact that analgesic activity of different substances in the writhing test could result from the stimulation of 5-HT_2 receptors while 5-HT_2 played a less important role (18, 27).

Peripheral 5-HT_2 and presynaptic 5-HT_{1A} receptors have been clearly shown to be involved in 5-HT-induced hyperalgesia (27). On the other hand, it is not excluded that 5-HT_{1A} receptors also played a role in this effect. In order to clarify the mechanism of action, we planned to determine analgesic activity of the test compounds, given together with antagonists of serotonin receptors. In addition, the involvement of serotonin receptors in the analgesic effect was supported by the fact that 5-HT when applied peripherally, was a potent proinflammatory and noxious agent, which caused hyperalgesia both in humans and rodents (27). 5-HT, released from platelets, mast cells, and basophils in injured or inflamed tissues, may play a role in inflammatory chemical milieu. Hyperalgesia, which is the major symptom of inflammation and tissue injury, probably caused by the phenylbenzoquinone, was the result of sensitization of nociceptors by a variety of inflammatory mediators. 5-HT is one of these mediators; indeed it has been shown be able to sensitize peripheral nerve fibers to other inflammatory mediators, such as bradykinin (27).

Next, we determined analgesic activity of the test compounds in another model of pain, namely the hot plate test. None of the tested xanthine derivatives prolonged the reaction time to a thermal stimulus. The proven activity in the writhing test with the concomitant lack of activity in the hot plate test indicates rather peripheral than central mechanism of analgesic action of the test compounds.

The next stage of research aimed to determine whether the test compounds, shown previously to possess antidepressant activity (12), can be used as co-analgetics. To answer this question, the compounds were tested for analgesic activity in the writhing test when administered in combination with ketoprofen.

The compounds slightly enhanced the analgesic effect of ketoprofen in the writhing test when given together.

In summary, compounds **1**-**4** were found to have analgesic effect when given alone, however, their effects differed in dependence on the pain model used. The analgesic activities were observed only in the writhing test. The strongest effect (similar to ketoprofen) was shown by compounds **3** and **4**. The possible mechanism of the antinociceptive effect of these compounds is thought to involve the activation of an analgesia mediated by serotonergic pathways or a combination of this mechanism with other important mediators playing a role in pain modulation. The compounds slightly enhanced the analgesic effect of ketoprofen in the writhing test when given in combination.

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BIOCHANIN A SHOWS NO EFFECT ON SKELETAL SYSTEM IN OVARIECTOMIZED RATS, WHEN ADMINISTERED IN MODERATE DOSE

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Abstract: Biochanin A is a naturally occurring isoflavone. Its main sources are clover species such as *Trifolium pretense*, *Trifolium subterraneum* or *Trifolium incarnatum*. Phytoestrogens, including isoflavones, are plant-derived substances, which exhibit estrogen-like properties, thus they may be used as an alternative for hormonal replacement therapies and prevent postmenopausal osteoporosis. Therefore, the aim of the presented study, was to investigate the effect of biochanin A on chemistry and mechanical properties of skeletal system in rats with ovariectomy-induced osteoporosis. The animals were divided into 4 groups – (I) sham-operated rats, (II) ovariectomized rats, (III) ovariectomized rats receiving estradiol at a dose of 0.2 mg/kg *p.o.*, which were a positive control, and (IV) ovariectomized rats receiving biochanin A at a dose of 5 mg/kg *p.o.* for four weeks. The administered dose of biochanin A is considered as moderate for human, which can be received in the dietary supplements, and was established using ten-fold conversion rate resulting from faster metabolism in rats. Obtained results showed that ovariectomy induced harmful changes in bone tissue, causing worsening in both chemistry and mechanical parameters in bones. Administration of biochanin A to ovariectomized rats did not affect any changes in bone tissue in comparison to the bones of untreated ovariectomized rats. There was neither improvement nor deterioration noted in chemical composition and mechanical properties in all analyzed bones. Basing on the results, it could be concluded, that biochanin A administered in a moderate dose shows no influence on bone tissue of rats with ovariectomy-induced osteoporosis.

Key words: biochanin A, ovariectomy, rats, osteoporosis, bones

Biochanin A (5,7-dihydroxy-4'-methoxy-isoflavone) is an isoflavone, which can be found in many clover species. In zigzag clover (*Trifolium medium*) it occurs in high concentration, and in red clover (*Trifolium pratense*), crimson clover (*Trifolium incarnatum*), haresfoot clover (*Trifolium arvense*), hungarian clover (*Trifolium pannonicum*) and red-feather clover (*Trifolium rubens*) its concentration is lower (1). It is also present in other plants such as soy, alfalfa, peanuts, and chickpea (2). This isoflavone is also reported in *Cassia fistula* and *Dalbergia odorifera* (3, 4). Biochanin A has been proven to have many pharmacological activities. Some *in vitro* studies indicated that this substance may act as a neuroprotective agent in L-glutamate-induced cytotoxicity in Parkinson's, Alzheimer's or Huntington's diseases (5), it is also an inhibitor of fatty acid amide hydrolase, the enzyme responsible for the hydrolysis of anandamide – the endogenous cannabinoid receptor ligand (6). It also inhibits allergic response in rat basophilic leukemia cells (7). There are reports that biochanin A shows antioxidative properties in cell cultures (8) as well as anti-inflammatory and anti-proliferative activities on RAW 264.7, HT-29 cell lines and mouse peritoneal macrophages (9). This isoflavone suppresses the proliferation of oral squamous carcinoma cells (10) and could be an anticancer agent which can selectively target cancer cells inhibiting multiple signaling pathways in HER-2-positive breast cancer cells (11). Moreover, it promotes apoptosis of prostate cancer cells (12, 13). Antiparasitic activity of biochanin A towards *Leishmania chagasi* and *Trypanosoma cruzi* is also confirmed (3). Furthermore, this flavonoid acts as antiviral agent in H5N1 influenza A virus-infected cells (14) and

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shows antimicrobial properties against *Clostridium* spp. (15).

What is more, there are *in vitro* and *in vivo* studies, indicating that biochanin A may inhibit melanogenesis, thus it can prevent the abnormal skin pigmentation in mice (16). This isoflavone was also examined *in vivo* in many animal models. Some investigations on rats show that biochanin A is an antifibrotic agent which prevents liver injuries (2, 17). Moreover, it shows antihyperglicemic effect (18), and can be used as chemoprevention of mammary tumorigenesis (19). Studies on murine xenograft model indicate that this substance may also inhibit the growth of the human breast cancer cells implanted into mice (20).

Isoflavones, including biochanin A, are considered as the phytoestrogens, due to their structural and functional similarity to 17β-estradiol. Phytoestrogens are plant-derived, nonsteroidal substances, which can bind to estrogen receptors (ER) and mimics the effect of estradiol, but their activity is lower when compared to 17β-estradiol (21). During menopause in women, endogenous estrogen level decreases, what leads to disadvantageous changes, such as flushing, but one of the most dangerous effect is osteoporosis (22). Hormonal replacement therapy (HRT) is used in postmenopausal women in order to reduce these symptoms, however this therapy is known to have some adverse effects on women's health (23). Therefore, to overcome negative effects of HRT and minimize symptoms of menopause, other substances, including isoflavones, have been tested. Until now, the effects of biochanin A on skeletal system have been examined in *in vitro* and *in vivo* studies. The *in vitro* experiment was conducted on mesenchymal stem cells and the results indicate that biochanin A prevents adipogenesis and enhances the differentiation of osteoblasts (24). Oral administration of this isoflavone to ovariectomized rats at a dose of 25 mg/kg for 14 weeks showed positive results in skeletal system (25). Even though the intake of phytoestrogens is commonly considered as a safe therapy, there are also some reports that long-term administration of higher doses of these substances may result in some complications, such as abnormal uterus bleedings or the increased occurrence of endometrial hyperplasia (26, 27, 28). Thus, in our study, the effect of biochanin A on bones in ovariectomized rats was investigated while administering this isoflavone at a dose of 5 mg/kg orally (*p.o.*) for 4 weeks.

MATERIALS AND METHODS

Virgin three-month-old Wistar female rats were provided by the Centre of Experimental Medicine at the Medical University of Silesia. Animals were fed with standard laboratory chow *ad libitum* and since the day preceding the experiment, the standard chow was replaced with chow containing no soybean. The research was carried out with the approval of the Local Ethics Commission in Katowice.

Rats were divided into four groups (n = 7): I – the control group of sham-operated, vehicle treated rats (SHAM), II – the control group of ovariectomized, vehicle treated rats (OVX), III – the ovariectomized rats receiving estradiol at a dose of 0.2 mg/kg *p.o.* (OVX + ES), and IV – the ovariectomized rats receiving biochanin A at a dose of 5 mg/kg *p.o.* (OVX + BioA). Administration of analyzed substances started one week after surgery and continued for 4 weeks.

All animals were weighted at the first day or research and after 4 weeks of the administration of

Table 1. Effects of estradiol and biochanin A on the body weight gain and weight of organs in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
Final body weight [g]	234.4 ± 4.3	245.8 ± 4.7	238.1 ± 6.4	246.4 ± 8.5
Body weight gain after 4 weeks [g]	15.9 ± 1.8	29.7 ± 2.4^{AA}	23.0 ± 1.7	37.6 ± 5.0
Uterus weight [g]	0.616 ± 0.085	0.103 ± 0.005^{AA}	0.252 ± 0.009^{BB}	0.100 ± 0.005
Thymus weight [g]	0.313 ± 0.018	0.482 ± 0.052^{AA}	0.500 ± 0.024	0.578 ± 0.048

Results are presented as the means ± SEM (n = 7). ^{AA} - $p < 0.01$ – statistically significant differences between the OVX and the SHAM groups; ^{BB} - $p < 0.01$ – statistically significant differences in comparison with the OVX group.

the substances. Moreover, the body mass gain was monitored during the whole experiment. One week before treatment with biochanin A and estradiol, sham surgery and bilateral ovariectomy were performed in general anesthesia induced by the mixture of ketamine and xylazine intraperitoreally. After 4 weeks of drugs administration, all animals were sacrificed with use of general anesthesia induced by ketamine and xylazine and cardiac exsanguination. Uterus, thymus and following bones: the right and left tibia and the right and left femur, as well as the L-4 vertebra were excised from each of the rats. The mass of analyzed bones was presented as the ratio of their mass per body mass determined after 4 weeks of the experiment.

The analysis of the mechanical properties of the bones

Investigation of the bone mechanical properties of the left femoral diaphysis, right tibial proximal metaphysis and right femoral neck was performed using the Instron apparatus, model 3342 500 N. Results were studied by using the software Bluehill 2, version 2.14.

For the femoral diaphysis, the bending test with three point loading was applied and following parameters were measured: the maximum load, the fracture load, displacement for maximum load, displacement for fracture load, and energy for maximum load. The Young's modulus and stress were also determined. Assessment of the mechanical properties of tibial proximal metaphysis was performed according to the method of Stürmer et al. (29, 30), using the three-point bending test. Likewise in femoral diaphysis, in tibial metaphysis: the maximum load, the fracture load, displacement for maximum load, displacement for fracture load, energy for maximum load, stress and Young's modulus were measured. The mechanical properties of the femoral neck were analyzed by performing a compression test and the maximum load affecting the femoral neck was determined. All methods used to examine the mechanical properties of the bones were described in previous studies (31, 32).

Assessment of the chemical content of the bones and the serum

Chemical content analysis involved the calculation of the water, organic substances and mineral substances content and the content of calcium and phosphorus in left femur (after bone mechanical properties analysis) and left tibia as well as in L-4 vertebra.

In order to determine the water content, the tibia, femur and L-4 vertebra were weighed after lyophilization in the lyophilizer Labconco Freezone 6 (USA) for seven days (temperature: -53^{o}C, pressure: 0.03 mBa). The difference between the bone mass obtained directly after the isolation and the bone mass determined after lyophilization corresponds to the water content.

Afterwards, the lyophilized bones were mineralized for 48 h in the muffle furnace type LG/11/C6 produced by Naberherm (The Netherlands) and weighed again. The bone ash obtained during the mineralization process comprised the mass of the mineral substances, and the difference between bone mass determined after lyophilization and bone mass after mineralization corresponds to the content of the organic substances in analyzed bones. The content of water, organic and mineral substances was presented as the ratio of water, organic and mineral substances mass per 100 mg of the bone mass after isolation.

In order to determine calcium and inorganic phosphorus content, mineralized bones were dissolved in 6 M HCI, diluted in distilled water, and then examined by the spectrophotometric method, using the kit Pointe Scientific, Inc. (USA) The content of calcium and phosphorus in the analyzed bones was presented as the ratio of the quantity of calcium and phosphorus per 100 mg of mineral substances.

In serum, the content of calcium and inorganic phosphorus was also determined. The assay was performed by spectrophotometric method using the kit Pointe Scientific Inc.

Statistical analysis

The results obtained during the tests were presented as the arithmetic means ± SEM.

The results were evaluated by one-way ANOVA, followed by Duncan's *post hoc* test. Nonparametric tests: Kruskal-Wallis and Mann-Whitney U test were performed, when necessary (lack of normality or of homogeneity of variance).

The differences were considered to be statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

The menopause is the mark of the end of natural female reproductive life and it is defined as the permanent cessation of menstruation resulting from the loss of ovarian follicular activity (33). The loss of the activity of ovarian follicular results in the decrease of endogenous estrogens. When such deficiency occurs, the rate of bone loss is accelerated – the osteoclasts' lifespan is increased and

osteoblasts' decreased. All these actions lead to postmenopausal osteoporosis (34). One of the most dangerous effects of postmenopausal osteoporosis are bone fractures. Due to the fact that the studies on bone mechanical resistance are unable to conduct on human, the animal model was developed. The best model for bone studies in postmenopausal period are ovariectomized rats. After ovariectomy, the osteoporotic changes similar to those in postmenopausal women are observed in these animals (35).

In our study, the ovariectomy proved to cause typical changes in rats. The body weight gain after 4 weeks in ovariectomized rats (OVX) was significantly higher than in sham-operated rats (SHAM) by 87.0%. What is more, the uterus weight was lowered by 83.2% and thymus weight increased by 53.9% (Table 1). Furthermore, weight of all analyzed bones was lower in OVX than in SHAM rats, but these changes were not statistically significant (Table 2). Estrogen deficiency also resulted in bone chemistry. The water content in analyzed bones increased, but the most significant changes were observed in femur and L-4 vertebra, where the water content was 9.9% and 11.8%, respectively, higher in OVX than in SHAM (Table 3). Furthermore, there was decrease of mineral content observed in tibia and L-4 vertebra by 4.4% and 8.9%, respectively, what is more, in L-4 vertebra, calcium content also decreased by 6.5% when compared to SHAM rats. Other studied parameters of bone chemical composition (organic fraction content as well as phosphorus content) were not affected by ovariectomy in statistically significant manner (Table 3). Ovariectomy also affected the mechanical properties of bones. The greatest changes were observed in tibia, where the following parameters were affected: maximal load, maximum stress, fracture load, energy for fracture load and stress for fracture load decreased by 43.9, 42.8, 44.5, 30.2 and 43.2%, respectively, in tibial metaphysis. Other parameters were not changed in statistically significant manner in all analyzed bones, however, the maximum stress and fracture stress were also lower in OVX group than in SHAM rats, both in femoral diaphysis as well as tibial metaphysis. There were no changes detected in femoral neck when comparing OVX to SHAM group (Table 5). Similar changes in ovariectomized rats were observed in our previous study (32), as well as in other studies (30, 36, 37).

In our experiment, the positive control was used. For this purpose, estradiol at a dose of 0.2 mg/kg *p.o.* was administered to ovariectomized rats. This substance is one of estrogens which can be used in HRT. HRT is used to overcome unfavorable effects of postmenopausal symptoms including osteoporosis (38, 39). Moreover, estradiol was also used in other studies as a positive control (30, 32, 36, 37). Ovariectomized rats receiving estradiol (OVX + ES) showed statistically insignificant decrease of body weight gain in comparison to OVX rats. What is more, in OVX + ES, the uterus weight increased by 147.7%, when compared to OVX group (Table 1). Macrometric parameters of all examined bones were not altered by administration of estradiol (Table 2). Chemical composition of analyzed bones was slightly affected by administration of estradiol at a dose of 0.2 mg/kg *p.o.* Except for mineral content in femur and L-4 vertebra, which increased by 4.3 and 6.0%, respectively, in OVX + ES when compared to OVX, other parameters in all

Table 2. Effects of estradiol and biochanin A on bone macrometric parameters in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMUR				
Weight [g]	0.663 ± 0.012	0.628 ± 0.006	0.631 ± 0.023	0.633 ± 0.023
Length [mm]	33.45 ± 0.27	33.50 ± 0.22	32.91 ± 0.25	33.37 ± 0.23
Diameter [mm]	3.25 ± 0.02	3.22 ± 0.04	3.20 ± 0.05	3.20 ± 0.05
TIBIA				
Weight [g]	0.517 ± 0.008	0.477 ± 0.014	0.489 ± 0.016	0.484 ± 0.015
Length [mm]	37.54 ± 0.34	37.59 ± 0.04	36.78 ± 0.19	37.01 ± 0.42
Diameter [mm]	2.63 ± 0.05	2.58 ± 0.05	2.62 ± 0.05	2.59 ± 0.03
L-4 VERTEBRA				
Weight [g]	0.176 ± 0.006	0.168 ± 0.011	0.163 ± 0.006	0.154 ± 0.005

Results are presented as the means ± SEM (n = 7).

Table 3. Effects of estradiol and biochanin A on bone mineral, H_2O, organic compounds, calcium and phosphorus content in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMUR				
H_2O content [mg/100 mg bone weight]	27.23 ± 0.74	29.92 ± 1.12^{A}	28.81 ± 0.52	30.47 ± 0.46
Organic compounds [mg/100 mg bone weight]	25.10 ± 0.69	24.87 ± 0.43	24.05 ± 0.17	24.13 ± 0.23
Mineral content [mg/100 mg bone weight]	47.67 ± 1.06	45.22 ± 0.81	47.14 ± 0.50	45.40 ± 0.55
Calcium content [mg/100 mg mineral substances]	39.43 ± 0.34	38.07 ± 0.94	39.55 ± 0.37	38.31 ± 0.45
Phosphorus content [mg/100 mg mineral substances]	15.79 ± 0.19	15.15 ± 0.37	15.55 ± 0.27	15.47 ± 0.17
TIBIA				
H_2O content [mg/100 mg bone weight]	25.60 ± 0.74	26.82 ± 1.16	26.32 ± 0.75	28.52 ± 0.75
Organic compounds [mg/100 mg bone weight]	25.89 ± 0.42	26.81 ± 0.34	26.24 ± 0.35	25.98 ± 0.47
Mineral content [mg/100 mg bone weight]	48.51 ± 0.43	46.37 ± 0.93^{A}	47.44 ± 0.52	45.50 ± 0.33
Calcium content [mg/100 mg mineral substances]	41.54 ± 1.53	38.03 ± 1.81	40.96 ± 0.52	41.79 ± 0.42
Phosphorus content [mg/100 mg mineral substances]	15.05 ± 1.04	14.66 ± 0.82	15.52 ± 0.26	16.00 ± 0.29
L-4 VERTEBRA				
H_2O content [mg/100 mg bone weight]	26.44 ± 0.24	29.56 ± 0.60^{AA}	27.66 ± 0.79	30.79 ± 0.58
Organic compounds [mg/100 mg bone weight]	26.29 ± 0.31	27.37 ± 0.73	26.58 ± 0.42	26.12 ± 0.33
Mineral content [mg/100 mg bone weight]	47.26 ± 0.37	43.08 ± 0.89^{AAA}	45.76 ± 0.66^{BB}	43.08 ± 0.37
Calcium content [mg/100 mg mineral substances]	43.78 ± 0.78	40.92 ± 0.65^{A}	41.06 ± 0.81	39.21 ± 0.61
Phosphorus content [mg/100 mg mineral substances]	16.71 ± 0.47	16.30 ± 0.45	15.96 ± 0.21	15.72 ± 0.21

Results are presented as the means ± SEM (n = 7). ^{A} - $p < 0.05$, ^{AA} - $p < 0.01$, ^{AAA} – $p < 0.001$ – statistically significant differences between the OVX and the SHAM groups; ^{BB} - $p < 0.01$ – statistically significant differences in comparison with the OVX group.

Table 4. Effects of estradiol and biochanin A on serum concentrations of calcium and inorganic phos-phorus in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
Calcium mg/100 mL]	8.99 ± 0.37	9.13 ± 0.20	9.43 ± 0.59	9.49 ± 0.45
Phosphorus [mg/100 mL]	5.45 ± 0.42	6.07 ± 0.49	7.55 ± 0.64	6.29 ± 0.36

Results are presented as the means ± SEM (n = 7).

Table 5. Effects of estradiol and biochanin A administered for 4 weeks on bone mechanical properties in ovariectomized rats.

Parameters	SHAM	OVX	OVX + ES	OVX + BioA
FEMORAL DIAPHYSIS				
Maximal load [N]	100.4 ± 4.8	99.9 ± 3.7	99.1 ± 5.1	101.1 ± 4.7
Displacement for maximal load [mm]	0.494 ± 0.020	0.546 ± 0.043	0.478 ± 0.030	0.591 ± 0.040
Energy for maximum load [J]	0.029 ± 0.002	0.032± 0.004	0.027 ± 0.003	0.036 ± 0.004
Maximum stress [MPa]	172.1 ± 26.3	116.6 ± 5.0	118.9 ± 2.9	121.0 ± 4.2
Fracture load [N]	100.1 ± 4.8	99.9 ± 3.7	99.1 ± 5.1	100.9 ± 4.7
Displacement for fracture load [mm]	0.497 ± 0.022	0.546 ± 0.043	0.478 ± 0.030	0.595 ± 0.041
Energy for fracture load [J]	0.030 ± 0.003	0.032 ± 0.004	0.027 ± 0.003	0.036 ± 0.004
Stress for fracture load [MPa]	171.5 ± 26.2	116.6 ± 5.0	118.9 ± 2.9	120.8 ± 4.2
Young's modulus [MPa]	6362 ± 274	5120 ± 325	5925 ± 268	5828 ± 358
TIBIAL METAPHYSIS				
Maximal load [N]	122.3 ± 4.2	68.6 ± 5.1^{AA}	85.7 ± 12.0	69.4 ± 3.1
Displacement for maximal load [mm]	0.947 ± 0.084	0.946 ± 0.039	0.840 ± 0.061	0.941 ± 0.066
Energy for maximum load [J]	0.065 ± 0.007	0.039 ± 0.004	0.044 ± 0.008	0.045 ± 0.004
Maximum stress [MPa]	85.4 ± 7.7	48.8 ± 7.6^{A}	70.2 ± 13.3	45.5 ± 3.6
Fracture load [N]	118.9 ± 4.6	66.0 ± 5.4^{AA}	84.9 ± 11.9	68.1 ± 2.9
Displacement for fracture load [mm]	1.042 ± 0.087	1.156 ± 0.080	0.886 ± 0.066	1.038 ± 0.057
Energy for fracture load [J]	0.076 ± 0.005	0.053 ± 0.005^{A}	0.049 ± 0.009	0.051 ± 0.003
Stress for fracture load [MPa]	82.8 ± 6.9	47.0 ± 7.4^{A}	69.6 ± 13.2	44.6 ± 3.3
Young's modulus [MPa]	3692 ± 818	2762 ± 1335	4009 ± 1135	2490 ± 807
FEMORAL NECK				
Maximal load (N)	84.0 ± 9.4	80.3 ± 6.8	84.3 ± 7.2	79.4 ± 4.9

Results are presented as the means ± SEM (n = 7). ^{A} - $p < 0.05$, ^{AA} - $p < 0.01$ – statistically significant differences between the OVX and the SHAM groups.

studied bones were not changed (Table 3). Administration of estradiol to ovariectomized rats did not affect mechanical properties of bones – all analyzed parameters remained unchanged (Table 5). There were no significant changes in serum calcium and inorganic phosphorus content in OVX + ES rats, when compared to OVX rats (Table 4).

Biochanin A was previously examined on bones *in vivo* in ovariectomized rats. The authors of the study administered biochanin A at a dose of 25 mg/kg *p.o.* to the animals for 14 weeks. Their results indicated that this isoflavone shows positive effect on rodents' skeletal system, improving the bone mineral density (BMD), bone mineral content (BMC) and the percentage of the trabecular bone volume in relation to the total tissue volume (25).

There are, however, some reports indicating that the usage of isoflavones at high doses and in long-time period could be unsafe for animals and human. Some studies show that the intake of this kind of phytoestrogens may lead to uterotropic changes and even to abnormal endometrial bleedings (26-28). What is more, Leclerc et al. indicate that phytoestrogens may interact with other receptors than ER (40).

For this reason, in our study, we administered lower dose of this isoflavone for 4 weeks. This dose was evaluated on the basis of commercially available pharmacological products named Promensil® and Promensil® Forte. In Promensil®, there is 26 mg of biochanin A per 40 mg of all isoflavones. Promensil® Forte has got double dose of isoflavones, when compared to Promensil® (41). Therefore, patients may receive biochanin A at doses of 26 or 52 mg. Considering the commonly used ten-fold conversion rate resulting from faster metabolism in rats, we administered biochanin A at a dose of 5 mg/kg to animals. Similar conversion was previously used in other study (42).

The treatment with biochanin A at a dose of 5 mg/kg *p.o.* showed no results on body weight gain, uterus weight and thymus weight in OVX + BioA rats when compared to OVX rats, however, there was statistically insignificant tendency of greater body weight gain after 4 weeks of treatment in OVX + BioA rats (by 37.9%), than in OVX rats (Table 1). Our results describing uterus weight overlap with those obtained by Su et al. (25). Also, the administration of this isoflavone did not affect any of the analyzed macrometric parameters of femur, tibia and L-4 vertebra in OVX + BioA rats in comparison to OVX rats (Table 2). The analysis of chemical composition revealed that administration of biochanin A did not cause any statistically significant changes in all examined bones in OVX + BioA rats when compared to OVX rats. Neither water content, organic content, mineral content nor the calcium and phosphorus content were altered (Table 3). What is more, the examination of mechanical properties also showed that biochanin A had no influence on studied bones in treated rats when compared to untreated animals (Table 5). Our results revealed that biochanin A administered at a dose of 5 mg/kg *p.o.* to ovariectomized rats shows neither beneficial nor harmful effect on skeletal system in laboratory animals. However, in previous studies, Su et al. reported that treatment with higher dose (25 mg/kg) in long-time period (14 weeks) shows advantageous effect on the rats' bones – it prevented the bone loss in ovariectomized animals. The authors examined bone mineral density, bone mineral content and bone volume. The serum calcium and inorganic phosphorus were also studied, but their levels were unchanged (25). In our study, the concentration of inorganic phosphorus and calcium in serum was also determined, and these results overlap with those presented by Su et al. (25) – there were no significant differences between all examined groups (Table 4).

There are many reports indicating that biochanin A is demethylated to genistein in digestive system. Such mechanism was observed in sheep, administered with ^{14}C-labeled biochanin A, where labeled genistein was observed in rumen fluids (43). *In vitro* studies conducted on human liver microsomes also showed, that biochanin A is metabolized to genistein and some conjugates by cytochrome P450 (44). Pharmacokinetics and metabolism of biochanin A was also studied in rats. Moon et al. (45) demonstrated that biochanin A in male rats after oral and intraperitoneal administration was demethylated to genistein and its conjugates. Similar observations were described by Singh et al. in female rats. The authors also determined the oral bioavailability of this isoflavone and considered this parameter as low. In this study, the authors also indicated that biochanin A is rapidly converted into free genistein, as well as into genistein and biochanin A conjugates (46).

Examining the effect of biochanin A on skeletal system, this isoflavone should not be considered as single agent influencing the bone tissue. Biochanin A in digestive system metabolizes *via* demethylation to other substances including genistein, which is also a potent agent affecting the bone metabolism. The majority of scientific reports suggests that genistein shows beneficial properties on bones, preventing from osteoporotic changes both in laboratory animals (including ovariectomized rats) and women suffering from postmenopausal osteoporosis (47, 48).

There are, however, some reports indicating that genistein shows disadvantageous effect on skeletal system. *In vivo* experiments conducted by Śliwiński et al. (49) proved that genistein has harmful effects on mechanical properties of bones and their chemical composition. There is also another study revealing that genistein displays neither beneficial nor injurious effect on skeletal system in rats (50).

Śliwiński et al. examined the effect of genistein on ovariectomized rats at a dose of 5 mg/kg; he also conducted *in vitro* experiment on mice osteoclasts applying doses of 1×10^{-9}, 1×10^{-8} and 1×10^{-7} M. In *in vitro* studies, the authors observed the advantageous effects of genistein revealed by the decreased osteoclasts number and reduced RANKL/OPG ratio. The best results in *in vitro* experiment were observed at a dose of 1×10^{-8} M. In spite of beneficial effect of genistein observed in *in vitro* study, the authors demonstrated that administering this isoflavone to ovariectomized rats at a dose of 5 mg/kg for 4 weeks fails to show any beneficial influence on bones. What is more, the administration of this isoflavone caused deterioration of some mechanical parameters e.g., maximal load in bones (49).

Another article focused on the effect of genistein on bone tissue in rats administered at low doses for long time period (5 months) also demonstrated that this isoflavone does not improve the microarchitecture of bones, regardless of the age of the animals and whether they had progeny or not. The authors examined two doses administered orally – 1.6 mg/kg and 3.2 mg/kg, and used three animal models: 3-month-old virgin rats, 12-month-old retired breeder rats and 14-month-old retired breeder rats. There were no statistically significant changes observed in any of analyzed groups which might suggest that genistein shows beneficial effect on BMC, BMD or histomorphological parameters in cortical and cancellous bone (50).

The above-mentioned examples of the effect of genistein on bone tissue, as well as the reports about pharmacokinetics of biochanin A may provide the explanation of the reason why this isoflavone administered at a moderate dose (converted to average dose taken by human) – 5 mg/kg for 4 weeks did not show beneficial effect on skeletal system in ovariectomized rats.

Biochanin A is one of the major constituents in red clover herb (*Trifolii pratense herba*). Studies focused on the effect of red clover extracts on osteoporotically changed bone tissue in ovariectomized rats proved that those extracts show beneficial effect on skeletal system (37, 51). Our previous study indicated that formononetin, which is also present in red clover, also shows the advantageous effect on bones in ovariectomized rats (32). However, Cegieła et al. concluded that the effect exerted by red clover is different and more complex than the one of estradiol itself, thus it could be assumed that not only isoflavones act as a preventive agents, but other constituents may affect skeletal system in rats (37).

CONCLUSION

Due to the fact that the biological activity of biochanin A depends on many factors such as metabolism and pharmacokinetics, it is very important to examine several doses of this substance. In our research we conducted the test investigating effect of biochanin A on skeletal system of rats with ovariectomy induced osteoporosis, using the dose, which can be assumed as moderate. This dose (5 mg/kg *p.o.*) was converted into the dose that can be intake by human in dietary supplements available in pharmaceutical market.

The results obtained during studies indicate that the administration of biochanin A at a dose of 5 mg/kg *p.o.* for 4 weeks to ovariectomized rats causes neither improvement nor deterioration of mechanical parameters of bones and their chemical composition.

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Conflict of interests

The authors state that they have no conflict of interests.

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CLINICAL EXPERIENCE OF LONG-TERM TREATMENT WITH ARIPIPRAZOLE (ABILIFY) IN CHILDREN AND ADOLESCENTS AT THE CHILD AND ADOLESCENT PSYCHIATRIC CLINIC 1 IN ROSKILDE, DENMARK

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Abstract: The aim of this paper is to share the clinical experience of the treatment of aripiprazole (Abilify) in children and adolescents. The authors have done a cross-sectional study about Abilify's treatment in children and adolescents with severe conduct problems (high impulsivity, aggression, outward reaction, physical cross-border behavior), high restlessness with ADHD, psychotic and psychosis-like symptoms with autistic disorders, psychosis, and intensive tics with Tourette's syndrome. The authors studied and described patients at the Child and Adolescent Psychiatric Clinic 1 in Roskilde, Denmark, who were treated with Abilify and were patients of the clinic in June 2013. The target group consisted of 33 patients, aged 9-18 years, which were in Abilify treatment during this time. Indications for the treatment and effectiveness of Abilify, Abilify's common doses used in children and adolescents, and the most common adverse effects of Abilify are presented.
Abilify was found to be effective, well tolerated and safe for children and adolescents. The dose depends on the complexity of diagnosis (higher doses used in cases of complex diagnosis), on the age (higher doses used in older children, but only in the case of noncomplex diagnoses). Statistical analysis shows that in cases of complex diagnoses, dosage does not depend on age but depends on other factors. It also shows that the effect of treatment is better for those who did not gain weight.

Keywords: Abilify, conduct disorder, autism

Second-generation antipsychotics are being used more often than ever before in children and adolescents with both psychotic disorders and a wide range of non-psychotic disorders. Several second-generation antipsychotics have received regulatory approval for some pediatric indications in various countries, but off-label use is still frequent (1).

Aripiprazole (Abilify) is an atypical antipsychotic partial agonist at dopamine D_2 and 5-HT_{1A} receptors and is an agonist at 5-HT_2 receptors. It has been described as a dopamine system stabilizer as, in high levels of dopamine production, it will act as an antagonist; and where dopamine activity is low, it will act as an agonist (2).

In Denmark, aripiprazole (Abilify) is licensed for treatment of schizophrenia, and treatment and prevention of recurrence of mania only for adults. In the USA, it was licensed by the Food and Drug Administration (FDA) in 2002 for the treatment of symptoms associated with autistic spectrum disorders in children and adolescents.

Although in Denmark, treatment of children and adolescents with Abilify is, as in many other European countries, as off-label use, this second generation, atypical antipsychotic is widely used in the treatment of psychiatric conditions in children and adolescents.

Starting around 2008, the Child and Adolescent Psychiatric Clinic 1 in Roskilde has used Abilify more widely and has gathered experience with it.

After five years of prescribing Abilify for children and adolescents, the authors have decided to share their experience and to describe patients who have undergone treatment with Abilify.

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The aim of our study is to share the clinical experience of the treatment of children and adolescents with Abilify.

MATERIALS AND METHODS

The authors have made a cross-sectional study about Abilify's treatment in children and adolescents. The authors have studied and described patients at the Child and Adolescent Psychiatric Clinic 1 in Roskilde, Denmark, who have tried treatment of Abilify and were patients of the clinic in June 2013. The patients received outpatient treatment at the clinic.

Most of the patients were still in treatment with Abilify at this point in time, but some of them have stopped Abilify treatment for different reasons (ineffectiveness of the treatment or unacceptable adverse effects).

Most of them started Abilify in the clinic, but some of them started the treatment in other places and later were referred to the clinic. Patients who

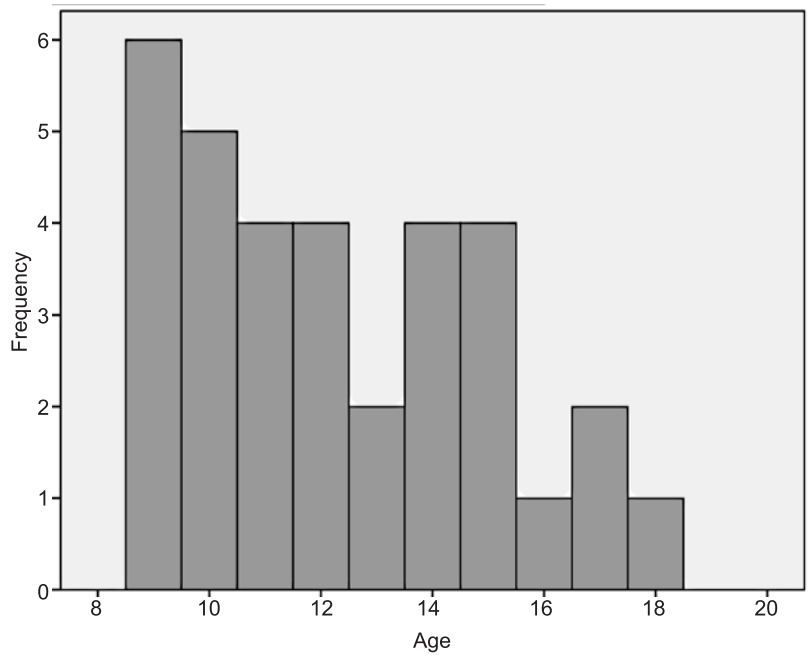

Figure 1. Age distribution in the study group

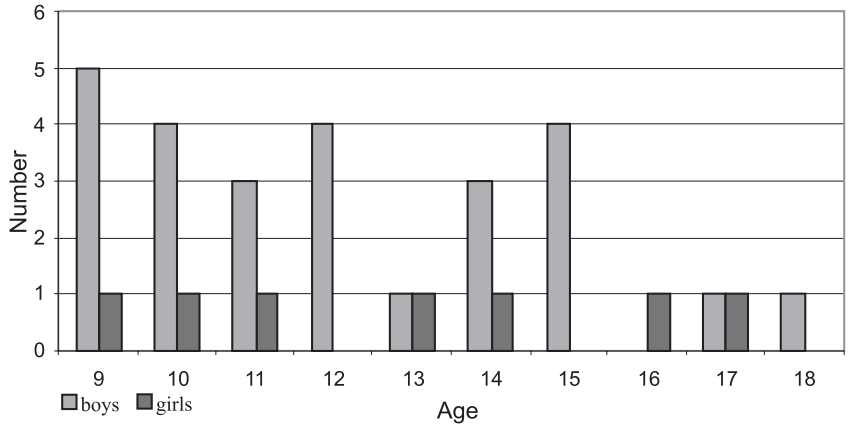

Figure 2. Age and gender distribution in the study group

received Abilify in the clinic starting in August 2008, but were discharged from the clinic at the start of June 2013, are not included in this study.

Another patient is not included in the group because of a short duration of the treatment (only one week). The treatment was interrupted because of intolerable adverse effects in the form of akathisia (the boy suffered inner restlessness, inability to sit still, and remained motionless); the dose and effect were not assessed.

Indications for starting Abilify's treatment for patients were the following:

- severe conduct problems (high impulsivity, aggression, outward reaction, physical cross-border behavior) with autistic disorder and ADHD;
- high restlessness with ADHD;
- intensive anxiety symptoms with autistic disorder;
- psychotic and psychosis-like symptoms with autistic disorders;
- psychosis;
- intensive tics with Tourette's syndrome.

The patients in the study group were divided into two subgroups: one of them was noncomplex and included patients diagnosed with ADHD, and the other group was complex and included patients with pervasive developmental disorders and psychosis, and patients who had two and more diagnoses.

The study group consisted of 33 children and adolescent psychiatric patients, 7 girls and 26 boys in ages 9-18, which have attended the Child and Adolescent Psychiatric Clinic 1 in Roskilde from August 2008 until June 2013, and have been in treatment with Abilify at least four weeks or longer (the longest period being almost five years).

Patients undergoing Abilify treatment were monitored with clinical psychiatric examinations with their parents. Physical examinations included weight control, blood pressure, blood tests, and EKG both before treatment and after, at approximately four weeks, six months, one year, and later once yearly.

Effectiveness of treatment for the psychiatric symptoms was assessed with only verbal reports from children and their parents about the child's functioning in school, at home, with peer groups, etc. Some of the adverse effects such as fatigue, akathisia, and increased appetite were also evaluated with only verbal reports from children and their parents. However, other possible adverse effects such as increased levels of cholesterol and prolactin were monitored with blood tests, along with other criteria (red and white blood cells, electrolytes balance, liver function tests, cholesterol and prolactin level). Weight gain as an effect of increased appetite was monitored with weight controls.

Finally, based on the data thus acquired, statistical analysis was performed. The following statistical methods were used: descriptive statistics (Figures 1-4, frequency tables 1-5) and inferential statistics (two independent samples *t*-test, Spearman rank correlation coefficient, Mann-Whitney U-test. Statistical package SPSS 21.0 was used for data processing).

Table 1. Diagnosis of the patients.

Diagnosis	Number of cases
Attention deficit hyperactivity disorder	28
Pervasive developmental disorders	13
Psychotic disorders	2
Tourette's syndrome	7
Attention deficit disorder	1
Anxiety disorders	2
Obsessive compulsive disorder	1
Adaptation disorder	1
Mental retardation	4
Inferioritas intellectualis	1
Other disorder of psychological development	1
Attachment disorder	1
Other tics disorder	1

Limitations

1. This study was not designed as a clinical trial, but rather a sharing of real, long-term, clinical experience.
2. The severity of symptoms and the effect of Abilify were not measured with any scale

Table 2. The mean daily dosage of aripiprazole in cases of complex and noncomplex diagnosis.

Complexity of diagnose	Number of patients	Mean daily dosage [mg]	Standard deviation
Noncomplex	11	1.75	1.30
Complex	22	6.27	3.34

Table 3. Adverse effects of Abilify in the study group.

Adverse effect	Number of patients
Only transient fatigue	3
Little tremor of hands in the start of treatment	1
Bothersome fatigue	1
Increase appetite with weight gain	11
Increase appetite with weight gain and transient fatigue	2
Hyperprolactinemia and increase appetite with weight gain	1
No adverse effect	14

Table 4. Adverse effects as a reason to interrupt the treatment.

Adverse effect	Number of patients
Bothersome fatigue	1 (3)
Increased appetite with weight gain	6 (18)
Hyperprolactinemia, increased appetite with weight gain	1 (3)

before or after the start of the treatment, but only with subjective reports about the effects on the symptoms from parents or patients.

3. Most of the patients have been in combined treatment, and in addition to Abilify, have been on the most common ADHD medicines such as methylphenidat and atomoxetin. Some of the patients have been on sertralin for anxiety and depression, and some of them on melatonin for sleep difficulty. Combined treatments were not taken into account.
4. Small sample size, considering number of variables in the study, which was not beneficent for using more sophisticated statistical methods, such like multiple regression analysis.

RESULTS

Description of the study group

Patients' psychopathology

The patients have been diagnosed with attention deficit and hyperactivity disorder (ADHD), pervasive developmental disorders, psychotic disorders, Tourette's syndrome, other tics disorder, anxiety disorders, adaptations disorder, mental retardation, attachment disorders, inferioritas intelectuallis, other disorder of psychological developmental.

But only 11 patients from the study group have been diagnosed with only one diagnose ADHD (F 90.0 or F 90.1), and most of the patients (22 patients or 70% of the patients) have been diagnosed with more complex psychiatric disorders such as pervasive developmental disorders or psychotic disorders, or they had ADHD in addition to other disorders such as Tourrette's syndrome, attachment disorder, mental retardation, anxiety disorder or other disorder of psychological development so their psychopathology is more complex. Later, the group was divided into subgroups of complex diagnosis and a subgroup of noncomplex diagnosis.

Age and gender of patients are given in Figures 1 and 2.

Doses used

The mean daily dosage of aripiprazole in the study group was 4.65 mg, standard deviation 3.52, a minimum dosage 0.25 mg and maximum dosage of 12.5 mg.

The mean daily dosage of aripiprazole in cases of complex diagnosis was 6.27 mg, standard deviation 3.34 and in cases of noncomplex diagnosis was 1.75 mg, standard deviation 1.30.

Tolerability, adverse effects and effectiveness of the treatment

There were 14 patients who had no adverse effects. Another 19 patients had such adverse effects as a fatigue, little tremor of hands in the start of treatment, increased appetite with weight gain, hyperprolactinemia.

There were six (18%) patients who complained of fatigue, and five of them (15%) had fatigue as a transient adverse effect. But in one case, fatigue was very bothersome and lasted as long as the boy was in treatment. There was, however, a positive effect on his impulsiveness and his intensive anxiety symptoms, but after five weeks the treatment was interrupted, because the boy was almost always tired and sleepy, making it impossible for him to function optimally.

Increased appetite with following weight gain was observed in 14 patients (42%). Increased appetite is a very serious adverse effect, and often for young people it becomes impossible to continue the treatment. Therefore, treatment was interrupted because of this adverse effect in 6 patients (18%).

Hyperprolactinemia was observed in only one patient. Unfortunately, the patient did not collaborate with a physician to have a blood test, and the boy developed gynecomastia - an obvious symptom

of hyperprolactinemia. His treatment was interrupted because of hyperprolactinemia and increased appetite.

The treatment was interrupted because of adverse effects in eight patients (24%).

However, there were 14 patients (42%) who had no adverse effects. If we put them together with other three patients (9%) who had transient fatigue and one patient (3%) who had little tremor of hands in the start of treatment, we can see that 18 patients could tolerate Abilify treatment and they had no adverse effect or only a very slight adverse effects. The largest part of the patients had good tolerability of Abilify (54%).

To defend Abilify as a safe antipsychotic medicine, it is necessary to point out that no Abilify-treated patient developed dyslipidemia, no patient had a cardiac adverse effect.

Of the patients treated, 21 (64%) had a positive effect, eight (24%) had some effect, only three (9%) did not have an effect, and only one (3%) had a negative effect of treatment (the patient reported deterioration of tics caused by Abilify).

Analyses of data

Correlations of the treatment's effect with used dosage, complexity of diagnosis, age, weight and weight gain were analyzed.

The authors also tried to analyze the factors that determined the doses. Are patients' age and weight or complexity of psychopathology the main determining factors for the choice of dose?

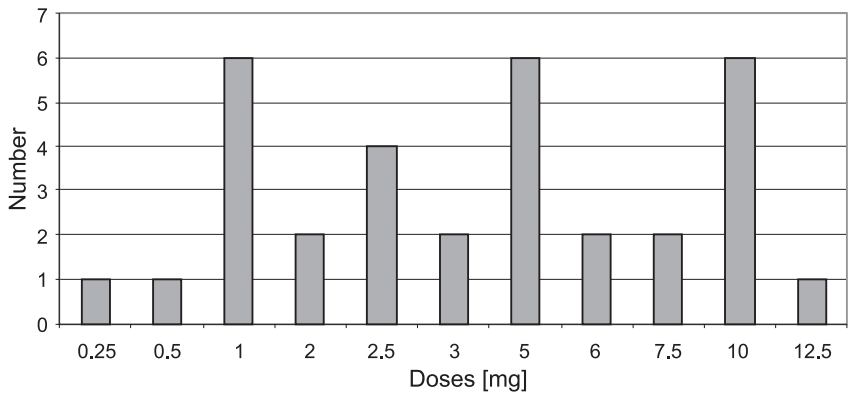

Figure 3. Doses used in the study group

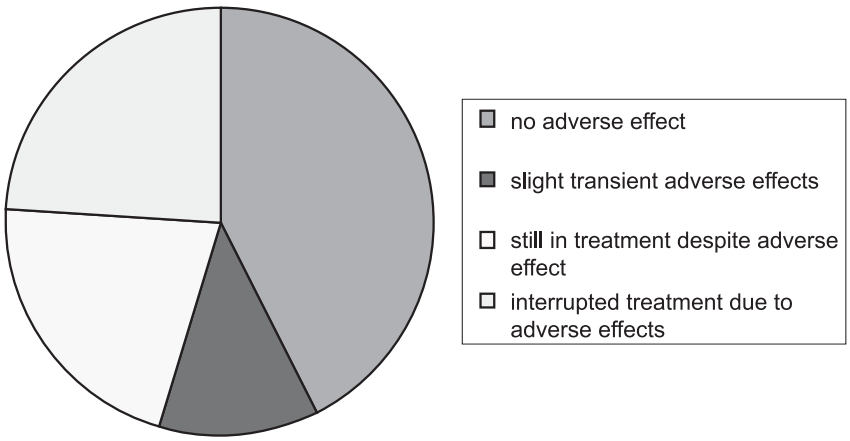

Figure 4. Tolerability of the treatment in the study group

Table 5. Diagnosis, ages, genders of the patients, doses used, and effects of the treatment in the study group.

No.	Complexity of psychopathology	Diagnosis	Doses [mg]	Effect	Age	Gender	Adverse effects	Still in treatment (blank) or reason of interruption of treatment
1	1	F 84.5	10	++	11	G	Fatigue	
2	1	F 90.0 F 84.0	6	+	11	B	Weight gain, increased appetite, fatigue	
3	1	F 20.9	10	++	10	B	Weight gain, increased appetite, fatigue	
4	1	F 90.0 F 84.5	5	++	17	G	Fatigue	
5	0	F 90.0	0.25	++	9	G	no	
6	1	F 90.0 F 70 F 43.23	10	++	18	B	no	
7	1	F 90.1 F 84.0	3	+	9	B	no	
8	0	F 90.0	2.5	+	12	B	Weight gain, increased appetite	Interrupted weight gain
9	1	F 90.1 F 84.5	5	++	15	B	Little tremor of hands in the start of treatment	
10	0	F 90.0	1	+	10	B	no	
11	0	F 90.1	1	++	9	B	no	
12	0	F 90.0 F 95.8	2.5	-	14	B	Weight gain, increased appetite	Interrupted weight gain ineffectiveness of treatment
13	1	F 84.0 F 42.0 F 90.0	5	0	13	B	Weight gain, increased appetite	Interrupted weight gain ineffectiveness of treatment
14	0	F 90.0	2	++	14	G	Weight gain, increased appetite	Interrupted weight gain
15	0	F 90.0	1	+	11	B	Weight gain, increased appetite	
16	1	F 90.0 F 95.2 F 84.5 F 41.1 F 40.2	12.5	++	9	B	no	
17	1	F 90.0 F 84.1	2.5	++	12	B	Strong annoying fatigue	Interrupted fatigue
18	1	F 90.1 F 88	5	+	14	B	Weight gain, increased appetite	Interrupted weight gain

Table 5. cont.

No.	Complexity of psychopathology	Diagnosis	Doses [mg]	Effect	Age	Gender	Adverse effects	Still in treatment (blank) or reason of interruption of treatment
19	1	F 98.8 F 95..2	2.5	++	17	B	no	
20	1	F 90.0 F 60.3	10	0	16	G	no	
21	1	F 90.0, F 84.0. F 95.2., F 70	5	++	14	B	no	
22	1	F 84.0 F 90.0 F 95.2	3	+	9	B	no	
23	0	F 90.0	1	++	10	B	no	
24	1	F 84.8 F 90.0 F 70.0	10	++	15	B	no	
25	1	F 84.0 F 90.1 F 95.2	10	++	12	B	no	
26	1	F 90.0 F 71	0.5	++	9	B	no	
27	0	F 90.0	1	++	10	G	Fatigue	
28	0	F 90.1	5	++	12	B	Weight gain increased appetite	
29	0	F 90.0	1	++	10	B	Weight gain, increased appetite	
30	1	F 90.0, F 95.2	7.5	0	15	B	Weight gain, increased appetite	Interrupted weight gain and ineffectiveness of treatment
31	1	F 28, F 94.1 R 41.8	6	++	11	B	Weight gain, increased appetite	
32	1	F 84.5	2	++	13	G	Weight gain increased appetite	
33	1	F 90.0, F 95.2 F 70	7.5	+	15	B	Weight gain increased appetite, hyperprolactinemia	Interrupted weight gain and hyperprolactinemia

++ positive effect, + some effect, 0 no effect, – negative effect, B = boy; G = girl

Statistical analyses of used doses show the following results:

1. Dose depends on the complexity of diagnosis. Higher doses are used in cases of complex diagnosis. Independent sample *t*-test shows that the mean dose is greater in cases of complex diagnoses ($p < 0.001$), standard deviation of dose is also greater for complex diagnoses ($p = 0.002$). Mann-Whitney U-test confirms results of the *t*-test, $p < 0.001$ (see Table 2).
2. Dose depends on age. Higher doses are used in older children. Spearman rank-correlation coefficient 0.42 shows that dose increases with an increase of age, $p = 0.015$ (statistically significant relationship).

   After separate statistical analysis of cases of complex diagnoses and noncomplex diagnoses, it appears that in cases of simple diagnoses, the dose depends almost exclusively on age ($p < 0.001$, Spearman coefficient 0.88), but in cases of complex diagnoses, dose does not depend on age ($p = 0.645$, Spearman coefficient 0.11) . It seems that dose depends on other factors.
3. Statistical analyses show that the effect of treatment is better for those who did not gain weight ($p = 0.040$, Spearman coefficient 0,11).

There were 84% cases with good effect for those who didn‘t gain weight; only 46% for those who did.

It seems that no weight gain gives better compliance and predicts a better result of the treatments.

DISCUSSION

As we mentioned before in the section of the limitations of the study, our study was not designed as a clinical trial. However, we observed the patients for a longer period of treatment than is done in a clinical trial, which usually lasts several weeks. Some of our patients have been in Abilify treatment for almost five years. Therefore, the observation of the patients for a longer period of treatment could be considered an advantage of our study.

The indications for starting Abilify's treatment for our patients have been similar as in other studies. In Ercan's study (3), there were: inattention, hyperactivity/impulsivity, delinquency, aggressive behavior, conduct disorder, oppositional defiant disorders.

In the study by Valicenti-McDermott and Demb (4), there were diagnoses within the autistic spectrum, mental retardation, attention-deficit/hyperactivity disorder/disruptive behavior disorders, mood disorders, reactive attachment and sleep disorders. Target symptoms included aggression, hyperactivity, impulsivity, and self-injurious behaviors.

In the study of of Masi et al. (5), target symptoms were Tourette's disorder and co-morbid ADHD.

As one can see, in our study group of 33 children and adolescents aged 9-18 years, we observed a positive effect in 64% of the patients. These are approximately the same results as in the studies by Ercan et al. (3). They conducted a study to determine the effectiveness and safety of aripiprazole in children and adolescents with both attention deficit/hyperactivity disorder and conduct disorder. In their eight-week, open-label study with 20 children and adolescents ranging in age 6-16 years they observed very much or much improvement with regard to inattention, hyperactivity/impulsivity, delinquency, aggressive behavior, conduct disorder, and oppositional defiant disorders in 63.1% of the patients.

Valicenti-McDermott and Demb (4) observed much improvement in regard to target symptoms in 56% of the patients in their study groups.

Results of a study by Findling et al. also suggest a clinically meaningful effect. Their study provides valuable safety and tolerability information regarding the long-term use of aripiprazole in the treatment of pediatric patients with irritability associated with autistic disorder (6). Moyal et al. showed a positive effect of Abilify for irritability and on quality of life (3).

We would also like to emphasize that mean daily dose in our study group is much lower than in Ercan's study (4.65 mg *versus* 8.55 mg).

Our results show that a higher dose is used in older children, which is also described in the study by Blumer et al. (8). Based on the results of the pharmacokinetic study, Blumer and colleagues (8) have proposed following weight-based dosing for pediatric patients: 1 mg for patients < 25 kg, 2 mg for patients between 25 and 50 kg, 5 mg for those between 50 to 70 kg , and 10 mg for patients with weight greater than 70 kg. It seems that larger doses for older children can be a consequence of their higher weight.

We can also see that the mean dose is greater in cases of complex diagnoses than in cases of noncomplex diagnoses. It could appear very logical: older children and those with more severe pathology need a higher dose of medications. However, our results also show that in cases of noncomplex diagnoses, the dose depends almost exclusively on age; but in cases of complex diagnoses, dose does not depend on age. It could be, that in cases of complex psychopathology, clinical dose depends more on the severity of pathology as well as on individual fac-

tors. It is well known that factors influencing choice of a treatment dose in a clinical situation can be many, they are inter-related, sometimes not easily measured, and therefore more sophisticated experiments and more advanced statistical methods (such like regression analysis) are desirable in order to explain treatment decisions of a physician. However, such methods require using considerably larger patient samples and possibly other research designs. This was not feasible in our situation.

In the studies of Ercan (3) and Masi et al. (5), no patient was excluded from the study because of adverse drug effects, but in our group, 24% of patients interrupted treatment because of adverse effects. We must take into account that there were patients who received treatment much longer that the patients from Ercan's eight-week, open-label study and Masi's 12-week open-label study.

Most studies report adverse effects from Abilify in the form of weight gain, increased appetite, fatigue, and tremors. We observed the same adverse effects.

Valicenti-McDermott and Demb (4) report adverse effects in 50% of the patients - almost the same as our results, where we observed adverse effects in 58% of the patients. As shown in their study, mean body mass index (BMI) rose significantly from 22.5 to 24.1. This is one of the most bothersome adverse effects, which complicated treatment with Abilify. In our study, increased appetite was observed in 42% of the patients.

In our study, the effect of treatment is significantly better for those who did not gain weight. It seems that no weight gain gives better compliance and predicts a better result of the treatments.

Many studies report that Abilify is a well tolerated, effective medicine with minimum extrapyramidal effects (9). Abilify does not show any adverse effect on QTc interval. Aripiprazole is not associated with increased prolactin or with dyslipidemia (10).

Our results show that there was only one patient in the study group, who had little tremor of hands in the start of treatment. During five years of the treatment, no Abilify-treated patient developed dyslipidemia, and only one patient developed prolactinemia. There were no patients who had a cardiac adverse effect.

Our experience and results should encourage clinicians to use Abilify in children and adolescents.

CONCLUSIONS

1. Abilify is found to be effective for child and adolescent psychiatric disorders (64% of the patients had positive effects, and 21% of them had some effects from the treatment).
2. Abilify is found to be well tolerated in children and adolescents. Fifty seven percent of the patients could tolerate Abilify treatment, and they did not have any side effects or had a very slight adverse effect.
3. Abilify is found to be safe. No Abilify-treated patient developed dyslipidemia or a cardiac adverse effect. Hyperprolactinemia and akathisia are seldom adverse effects of Abilify.
4. Increased appetite and weight gain are the most common adverse effects of Abilify. Increased appetite and weight gain were observed in 42% of patients.
5. The dose depends on the complexity of diagnosis and on the age. Higher doses are used in cases of complex diagnosis. Higher doses are used for older children, but only in the case of noncomplex diagnoses. Statistical analysis shows that in cases of complex diagnoses, dosage does not depend on age.
6. Statistical analysis shows that the effect of treatment is better for those who did not gain weight.

Declaration of interest

The authors do not have any interests to declare.

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GENERAL

RATIONALIZED AND COMPLEMENTARY FINDINGS OF SILYMARIN (MILK THISTLE) IN PAKISTANI HEALTHY VOLUNTEERS

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Abstract: The aim of the work was to examine the influence of gender on pharmacokinetics of silymarin; a basic constituent of medicinal herb "milk thistle" (*Silybum marianum*). The presented work is the extension of published work of Usman et al. (16). The comparative parallel design pharmacokinetic study was conducted in Pakistani healthy volunteers (male and female) receiving a single 200 mg oral dose of silymarin. Sixteen subjects (8 males and 8 females) were enrolled and completed the 12 h study. Blood screening was done on HPLC and the pharmacokinetic parameters were calculated by APO, 3.2 Ver. software using non-compartmental and two compartment model approaches. A significant difference ($p < 0.05$) was observed in almost all calculated pharmacokinetic parameters of silymarin in male and female. Clinically, the silymarin has been underestimated in the previous study. Gender based clinical investigations should be directed in the future on other flavonolignans of 'milk thistle' as well.

Keywords: gender-based pharmacokinetics, milk thistle, parallel design, silymarin, two compartment model

There is a general belief amongst the consumers all over the world that herbal drugs are always safe because they are natural or near to nature but evidence suggests otherwise. In Pakistan, traditional healers (Hakims/Tabibs) are registered by the government under an Act of the Parliament but there is no regulatory control on the manufacture, sale, distribution etc. of traditional medicines (1). Requirements and methods for research and evaluation of the safety and efficacy of herbal medicines are more complex than those for conventional pharmaceuticals. A single medicinal plant may contain hundreds of natural constituents, and a mixed herbal medicinal product may contain several times that number. If every active ingredient would be isolated from every herb, the time and resources required would be tremendous. Such an analysis may actually be impossible in practice, particularly in the case of mixed herbal medicines (2). Female and male have different body compositions. The body fate percentage is larger and the body water content is smaller in female. Furthermore, these differences are age dependent, with body fate increasing in both genders with age. Body fat composition may affect the volume of distribution of many drugs. For lipophilic drugs such as opioids and benzodiazepines, the volume of distribution per kg body weight generally will be higher in females than in males. Conversely, the volume of distribution for water soluble drugs such as muscle relaxant may be lower in females than in males. Thus the same dose per kg body weight will result in a lower

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initial plasma concentration of lipophilic drugs in females, whereas the initial concentration of water soluble drugs will be higher (3). Generally, males weigh more than females, yet dosing of most drugs is not corrected for body weight. For the drugs evaluated by the FDA in the bioequivalence studies, not adjusting for weight resulted in 20%–88% higher AUCs in females compared with males in the dataset where there was a significant sex difference. For drugs with narrow therapeutic ranges or steep dose-concentration curves or both, this may cause significantly increased adverse events in females compared with males. Even within a group of females, not taking into account body weight can affect efficacy. Holt et al. (4) found that if a woman weighed >70.5 kg, she had a 1.6 greater risk of oral contraceptive (OC) failure. With a low-dose OC and weight >70.5 kg, the relative risk (RR) increased to > 4-fold (5). Milk thistle (*Silybum marianum* (L.) Gaerth) is an annual wintering plant belonging to Asteraceae family that reaches a height of 200-250 cm. The capitula are 5-8 cm in diameter, and ovate. The flowers are purple in color. The sunny, stony slopes of the Mediterranean region are the growing locations of warmth loving milk thistle. It is common in countries of Mediterranean region. The ripe fruit of milk thistle contains flavonoids. Silymarin, a flavonolignan from 'milk thistle' (*Silybum marianum*) plant is used from ancient times as a hepatoprotective drug. Along with hepatoprotective action, other actions include antioxidant, anti-lipid peroxidative, antifibrotic, anti-inflammatory, immunomodulatory and liver regenerating. Silymarin has clinical applications in alcoholic liver diseases, liver cirrhosis, Amanita mushroom poisoning, viral hepatitis, toxic and drug induced liver diseases, psoriasis, and has neuroprotective and neurotropic activity. The seeds of milk thistle contain approximately 70-80% silymarin flavonolignans and approximately 20-30% of chemically undefined fraction, composed of mostly polymeric and oxidized polyphenolic compounds. Silymarin is a complex mixture of four flavonolignan isomers, namely silybin, isosilybin, silydianin and silychristin with an empirical formula $C_{25}H_{22}O_{10}$. Among the isomers silybin is the major and most active component and represents about 60-70%, followed by silychristin (20%), silydianin (10%), and isosilybin (5%). The seeds also contain betaine, trimethylglycine and essential fatty acids that may contribute to silymarin's hepatoprotective and anti-inflammatory activities (6, 7). Their mechanisms of action are still poorly understood. However, the data in the literature indicate that silymarin and silibinin act in four different ways: (i) as antioxidants, scavengers and regulators of the intracellular content of glutathione; (ii) as cell membrane stabilizers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes; (iii) as promoters of ribosomal RNA synthesis, stimulating liver regeneration; and (iv) as inhibitors of the transformation of stellate hepatocytes into myofibroblasts, the process responsible for the deposition of collagen fibers leading to cirrhosis. The key mechanism that ensures hepatoprotection appears to be free radical scavenging. Anti-inflammatory and anticarcinogenic properties have also been documented (8). The chemoprotective action of silymarin opened the newer application of silymarin in the field of cancer therapy. The incidence of urinary bladder neoplasms and preneoplastic lesions induced by N-butyl-N-(4-hydroxybutyl)nitrosamine were significantly reduced. Silymarin also significantly inhibited azoxymethane induced colon carcinogenesis in rats. Skin carcinogenesis induced by benzoyl peroxide or 12-tetradecanoylphorbol-13-acetate was also inhibited by silymarin (9). Noteworthy, there is recent evidence of the inhibition of hepatitis C virus (HCV) RNA polymerase by silymarin and results from several small clinical trials suggest that silymarin could be used as an adjunctive therapy for HCV infection (10). It may also help prevent toxin entry into cells or possibly be involved with toxin exportation. Its purported mechanism of hepatoprotection may also include modulation of both phase I and phase II detoxification pathways in a dose dependent manner. In *in vivo* mice models, silymarin was shown to stimulate the phase II detoxification pathway, increasing levels of glutathione and glutathione S transferase, in a dose dependent manner in several tissues, including liver, lung, stomach, small bowel, and skin (11). The bioavailability of enterally administered silymarin is limited; the compound is poorly soluble in water, and only 20-50% is absorbed from the gastrointestinal tract after ingestion. Absorption is significantly enhanced if silybin is administered in a complex with phosphatidylcholine. There is rapid absorption after an oral dose with the peak plasma concentration reached after two to four hours and an elimination half-life of six hours it undergoes extensive enterohepatic circulation. Three to eight percent is excreted in the urine, and 80% is excreted in the bile as glucuronide and sulfate conjugates. Bioavailability can vary up to three-fold depending on the formulation; the brand used in most European studies, Legalon® contains approximately twice as much available silybin as other preparations (12). Low water solubility (0.04 mg/mL) of silymarin is report-

ed. Solubility of silymarin in various other solvents like transcutol, ethanol, polysorbate 20, and glycerylmonooleate is 350.1, 225.2, 131.3 and 33.2 mg/mL, respectively. Silymarin possesses no lipophilic properties, even though its water solubility is poor (13). Apart from the role the physicians have to play in safeguarding the public health, pharmacist's interventions in the appropriate use of herbal medicines are necessary to make the overall health delivery system safe and effective. Pharmacists should therefore be knowledgeable about the medicinal plants, herbal therapies and other herbal based dietary supplements in view of their increasing popularity and utilization so as to be able to provide objective information to the consumers (1, 14). Major activity of Food and Drug Administration (FDA) Office of Women's Health (OWH) is ensuring the development of consistent regulatory policies relating to the participation of women in clinical trials, and analysis of the data to detect gender differences. Whenever appropriate, OWH ensures that this information is incorporated into product labeling (15). The presented work is the extension of Usman et al. study (16). In the previous work, pharmacokinetic study was conducted only on male. In the present work, both the gender, male and female, were included in the study to encompass any difference in pharmacokinetics of silymarin due to gender variation.

MATERIALS AND METHODS

Materials

Silymarin standard (98%, HPLC) was provided by Abbot Pharmaceuticals Pvt. Ltd. Karachi, Pakistan. Methanol (96%, HPLC), potassium dihydrogen phosphate (98%, analytical) and phosphoric acid (85%, analytical) were obtained from Merck, Germany. Silymarin 200 mg tablets manufactured by Abbott Pharmaceuticals Pvt. Ltd. Karachi, Pakistan were purchased from the local market. All reagents and solutions throughout the research were prepared in fresh stock (not older than one week) of distilled water.

Instruments

Water was distilled by Water Distillation Apparatus (Köttermann). The pH of distilled water was confirmed *via* Cyber Scan pH meter before and after use. The analysis was carried out using HPLC (Shimadzu LC.9, CSW 32 Ver. 1.3 Software, Japan) equipped with a degasser, quaternary pump, manual sampler and a UV detector (Shimadzu SPD-6AV) connected to data collection system.

Chromatographic conditions

The analytical column was a Brownlee MPLC 5 µm (220 × 4.6 mm, pore size 80 Å) with packing C18 (RP18, ODS, Octadecyl) from Perkin Elmer, maintained at ambient room temperature. The extraction and emission wavelength were adjusted at 288 nm.

Preparation of mobile phase

The mobile phase was prepared by dissolving 2.72 g of potassium dihydrogen phosphate in methanol quantity sufficient to make final volume of 1 L with pH 2.8 adjusted with phosphoric acid and filtered through 0.45 µm membrane filter prior to use.

Preparation of standard and working solutions

Working solutions were prepared in mobile phase by 0.3, 0.6, 1.25, 2.5, 5 and 10 µg/mL dilutions. A stock solution of silymarin standard was prepared freshly by dissolving 50 mg drug in 50 mL of methanol to give a final concentration of 1 mg/mL.

Extraction method

The extraction procedure and other factors were kept constant to see the influence of gender only on pharmacokinetics of silymarin. A hundred milliliters of human blood sample was collected from blood bank of UVAS. The plasma was separated from blood by centrifugation (17). The extraction procedure was carried out as described by Usman et al. (16). Briefly, 100 µL of acetate buffer at pH 5.6 and glucuronidase type HP-2 (30 µL) (Helix pomatia, Merck, Germany, 127300 units/mL) were added to a 100 µL of plasma sample and this mixtures were incubated at 37^{0}C for 2 h with periodical shaking. Then, 200 µL of borate buffer (pH 8.5) and 2 mL of diethyl ether were added to the mixture. The mixture was vortexed for 1 min and centrifuged at 3000 rpm for 2 min. Then, organic phase was transferred into a sample test tube and evaporated under nitrogen steam. The residue was reconstituted in a 130 µL aliquot of the mobile phase, vortexed for 30 s and centrifuged for 1 min at 2500 rpm, and then 100 µL of the solution was injected directly into the chromatographic system. The same extraction method was implemented on blood samples of the test subjects.

Subjects and materials

Sixteen healthy Pakistani volunteers (8 male and 8 female) ranging from 18 to 45 years and from 50 to 80 kg in age and weight, respectively,

were selected. Alcoholic, drug abused, hepatitis B & C positive, pregnant and known history of hypersensitivity with the drug under study volunteers were rejected according to the inclusion and exclusion criteria of therapeutic ethical committee of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. Additionally, the volunteers who had bleed and/or taking any medicine within three months before initiating the study as well as who were incapable of understanding written consent were also abandoned from the study. Written informed consent was taken from each volunteer who received a dose of silymarin 200 mg orally. Each subject was fasted after midnight (00:00 a.m.) before the administration of the drug in next morning (9:00 a.m.). The subjects continued to fast for 3 h after administration. Volunteers were housed at blood collection center throughout the period of blood sampling. Eight samples of 5 mL blood each were collected over a period of 12 h from each subject at predefined time schedule of zero, 0.5, 1, 2, 3, 5, 8 and 12 h after silymarin administration and stored in heparinized glass tubes. The plasma was harvested from blood cells by centrifugation and stored at -40^{0}C until analysis.

Pharmacokinetics and statistics

The absorption and elimination kinetics profile of silymarin was determined by software APO PC-Computer Program, MWPHARM version 3.02, MEDIWARE, Holland. The program determines compartmental and non-compartmental analysis in calculation of the bioavailability and elimination kinetic parameters. GraphPad Prism 5 was used to apply unpaired *t* test for statistical analysis of the data.

Validation and optimization

Some parameters tested during the validation process were: system suitability, selectivity and linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision.

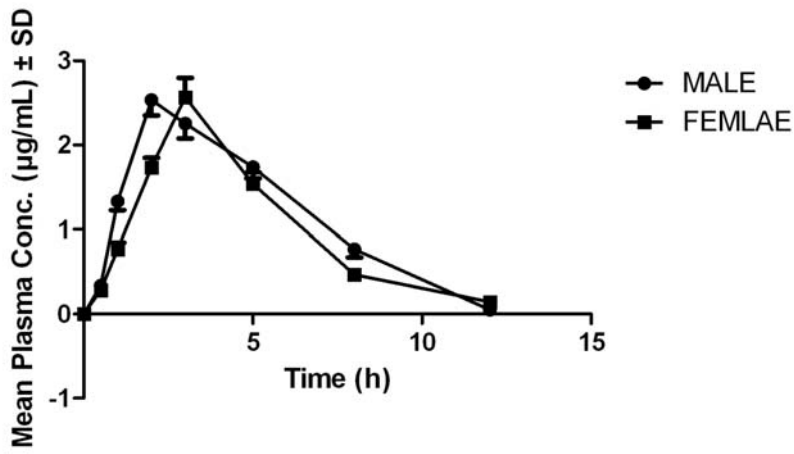

Figure 1. Mean plasma concentrations ± SD and time profile of healthy male and female volunteers

Table 1. Demographic data for volunteers (n = 8).

	Males				Females			
	Age (year)	Weight (kg)	Height (cm)	BMI	Age (year)	Weight (kg)	Height (cm)	BMI
Minimum	19	50	149	22	19	50	145	22
Maximum	29	68	169	24	27	62	162	25
Mean	23	58	158	23	23	56	154	24
SD*	3.6	6.3	7.8	0.81	2.4	4.6	6	1.1
CV**	15.50%	10.91%	4.93%	3.50%	10.46%	8.27%	3.87%	4.82%

*SD = standard deviation, **CV = coefficient of variance.

Table 2. Validated performance of treated samples (mean ± SD).

Parameters	Properties test	No. of treatment	Test conditions	Results	Acceptance criteria	Remarks
System suitability	Retention time (Tr) (min)	n > 3	Normal (predefined) and stress study (accelerated stability study)	[silychristin, silydianin, silybin A and silybin B] [4.62 ± 1.19 (%RSD 0.069), 12.09 ± 0.9 (%RSD 0.37), 12.32 ± 2.1 (%RSD 0.76), 14.4 ± 3.4 (%RSD 0.37, respectively)]	RSD = 2.0%	No interfering peaks
Selectivity and linearity	Goodness of fit test	n > 3	predefined	y = 13.87x + 5.7505, r^2 = 0.999	r^2 > 0.9	Results were reproducible within the stated range
LOD and LOQ (µg/mL)	Qualification and quantification	n > 5	predefined	0.036 ± 0.01, %RSD 7 0.06 ± 0.005, %RSD 0.7, respectively	%RSD ≤ 1.0%	-
Precision (% CV) and accuracy (%)	Intra-day and inter-day variations	n > 5	predefined	1.2-9.5%, ≥ 93%, respectively	% CV ≤ 10%, 85-105%	Higher % CV (≥ 10%) was observed at lower conc. (0.03 µg/mL)

Stability study

Chemical stability of silymarin in plasma was assessed in accordance with the Guidelines for industry: Bioanalytical method validation (18) (results were not shown).

RESULTS

The objective of proceeding extraction procedure of Usman et al. (16) was to encompass the influence of gender only. The demography of the volunteers has been shown in Table 1. The bioanalytical method was validated prior to quantify the drug in subject samples. Extraction efficiency (EE) of silymarin as silychristin, silydianin, silybin A and silybin B (chromatogram was not shown) was measured by expression: .

$$E.E = \frac{\Sigma(AUC)_{Extracted}}{\Sigma(AUC)_{Non\text{-}extracted}} \times 100$$

The % recovery (EE) was 89-103% with bias -13 to +17 at stated concentrations. Other parameters of validation process are given in Table 2. The results of the developed method were revalidated after one month and inter-day precision was defined. The mean plasma concentration ± SD *versus* time profiles of silymarin for the gender (male and female) is shown in Figure 1. The absolute percentage difference between the means of calculated pharmacokinetic parameters of male and female was 9.52-100. The mean values of time for maximum concentration (T_{max}), half lives (absorption, phase I and phase II), volume of distribution (V_d), volume of distribution in compartment 1 (V_{di}), volume of distribution steady state (V_{dss}), clearance (CL) and mean residence time (MRT) were higher in females while the values of peak plasma concentration (C_{max}), area under curve (AUC), AUC polyexponential, AUC trapezoidal, absorption rate constant (k_a), elimination rate constant from compartment 1 (k_{10}), transfer constants (k_{12} and k_{21}) and lag time were lower in females as compared to those in males (Table 3). There was significant difference ($p < 0.05$) in all calculated pharmacokinetic parameters between the male and female subjects except transfer constant k_{12} ($p > 0.05$). High significant difference ($p = 0.0001$) was observed in C_{max}, V_{di}, V_{dss} and AUC, especially if calculated by trapezoidal rule. If

we compare the result with those of Usman et al. (16), from minor to major variations were observed in the reported pharmacokinetics parameters (Table 4). In comparison with Silliver®, the results of T_{max} and AUC increased ~13% to ~19%, respectively, in male. A decline was observed in the values of C_{max}, CL, MRT and V_d and amounted: ~4, ~11, ~16 and ~62%, respectively. Comparing the results with Silimarin®, AUC, T_{max} and C_{max} were increased by ~15, ~19 and ~47%, respectively, while the results of CL, MRT and V_d were reduced by ~9, ~37 and ~69%, respectively, in male. On the other hand, in comparison of female with Silliver®, an increment of ~4 and ~37% was observed in AUC and T_{max}, respectively, in female. The results of MRT and CL were also increased (~5%) comparatively in female. A decline of ~36 and ~41% were observed in C_{max} and V_d, respectively, in female. Comparing the results with Silimarin®, CL and T_{max} were increased by~7 and ~44%, respectively, in female. The results of MRT and V_d were decreased ~23 and ~53%, respectively, in female. Zero to negligible change (<

Table 3. Pharmacokinetics of silymarin for male and female volunteers (mean ± SD).

Parameters	Males	Females	p value	Results
Peak concentration (C_{max}) [µg/mL]	2.79 ± 0.35	1.86 ± 0.12	< 0.0001	*sig
Time to peak (T_{max})	2.14 ± 0.26	2.6 ± 0.12	0.0004	*sig
Area under curve (AUC) [µg×h/mL]	12.82 ± 0.77	11.27 ± 1.01	0.004	*sig
AUC polyexponential (t = 12)	12.79 ± 0.75	11.21 ± 0.99	0.0029	*sig
AUC trapezoidal rule (t = 12)	14.23 ± 0.68	12.08 ± 0.94	0.0001	*sig
Half life phase I	1.19 ± 0.2	1.53 ± 0.09	0.0006	*sig
Half life phase II	1.19 ± 0.2	1.55 ± 0.1	0.0006	*sig
Absorption half life	1.19 ± 0.2	1.54 ± 0.1	0.0006	*sig
Absorption rate constant (k_a) [1/h]	0.6 ± 0.09	0.45 ± 0.03	0.0009	*sig
Rate constant (k_{10}) [1/h]	0.6 ± 0.09	0.45 ± 0.03	0.0009	*sig
Rate constant (k_{12}) [1/h]	0.75 ± 2.11	$2.53e^{-005}$ ± $7.10e^{-005}$	0.3343	**ns
Rate constant (k_{21}) [1/h]	0.6 ± 0.09	0.45 ± 0.02	0.001	*sig
Volume of distribution (V_d)	26.74 ± 3.6	39.74 ± 2.7	< 0.0001	*sig
Volume of distribution in compartment 1 (V_{di})	26.74 ± 3.6	39.68 ± 2.71	< 0.0001	*sig
Volume of distribution steady state (V_{dss})	26.74 ± 3.6	39.68 ± 2.71	< 0.0001	*sig
Clearance (CL) [l/h]	15.66 ± 0.89	17.87 ± 1.66	0.0051	*sig
Mean residence time (MRT)	3.85 ± 0.55	4.83 ± 0.27	0.0005	*sig
Lag time	0.42 ± 0.04	0.38 ± 0.02	0.024	*sig

*sig = significant, **ns = not significant.

Table 4. Comparison of pharmacokinetics of silymarin (200 mg orally administered in male and female) with published work of Usman et al. (16).

Present study			Usman et al. (16)	
Parameters	Silymarin 200 mg		Silliver®	Silimarin®
	Male	Female		
Peak concentration (C_{max}) [µg/mL]	2.79	1.86	2.9	1.9
Time to peak (T_{max}) [h]	2.14	2.6	1.9	1.8
Area under curve (AUC) [µg×h/mL]	12.82	11.27	10.8	11.2
Mean residence time (MRT) [h]	3.85	4.83	4.6	6.1
Volume of distribution (V_d) [L/kg]	0.46	0.71	1.2	1.5
Clearance (CL) [mL*h/kg]	270	319.11	303.5	297.4

1%) was observed in case of C_{max} and AUC, respectively, in female.

DISCUSSION

Numerous studies have shown a gender difference in the pharmacokinetics of many drugs. Pharmacokinetic differences arise because of differences in endogenous and exogenous hormones, differences in body size and fat compositions, and difference in liver metabolism. Approximately 50% of the drugs currently on the market are metabolized by cytochrome P-450 isozyme 3A4.Women appear to have higher levels of 3A4 than men (15, 19). The results of accuracy and precision showed that the extraction method was accurate and reproducible for plasma concentrations. It was inferred from the statistical analysis that pharmacokinetics of silymarin behaved differently in male and female that might be due to reduced liver blood flow and lower clearance of the drug in female volunteers as compared to male (3). As silymarin was the lipophilic extract of "milk thistle" that made it a potential candidate to reach in deep tissues of the body, especially in females that had high fat content as compared to male. This factor was led to increase of ~12, ~18, ~20, ~23 and ~33% in mean values of CL, T_{max}, MRT, half lives (phase I and phase II) and volumes of distribution (V_d, V_{di}, V_{dss}) in female (Table 2) (20, 21). Under very general assumptions, the area under the plasma or blood drug concentrations is a parameter that is closely dependent on the drug amount that enter into the systemic circulation and on the ability that the system has to eliminate the drug (clearance) (22). On the basis of higher mean values of AUC and C_{max} (12% and 33%, respectively) in male as compared to female, it is expected that much better bioavailability of silymarin will be achieved in male. As much of the drug was transferred from central compartment to deep tissue in female because of high fat contents that led to reduced amount to reach systemic circulation and resulted in low AUC and C_{max} in female as compared to male. The rate of absorption and distributions (k_a, k_{10}, k_{12} and k_{21}) was high in male as compared to female that might be due to difference in life style of Pakistani males and females. The ratio of fast food intake, use of tea, exercise etc., is high in Pakistani males as compared to females. All these factors directly influence the function of GIT. Two compartment model approach was used in this study to calculate pharmacokinetic parameters as compared to the previous study of silymarin by Usman et al. (16). The variation in pharmacokinetics of silymarin in comparison with the previous study might be due to demographic difference in gender.

CONCLUSIONS

On the bases of observed variations in pharmacokinetics of silymarin in male and female it will be right to say that gender based evidence should be provided if clinical study is reported especially on human being. It is evident that the dose of silymarin must be adjusted prior to administration according to demographic parameters of the patient. Furthermore, the presented work demands further research to conduct on larger population in the future to make a concrete decision about the dose adjustemnt of silymarin in male and female. It is also recommended by the authors that *In Vitro In Vivo* Correlation (IVIVC) shoud be developed for silymarin to predeict bioavalibity of the newly developed formulatons (23, 24).

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Conflict of interest

The authors declare that there is no financial support and conflict of interest related to the content of this manuscript.

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KAP AMONG DOCTORS WORKING IN HOSPITALS, REGARDING HALAL PHARMACEUTICALS; A CROSS SECTIONAL ASSESSMENT

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Abstract: There is a growing awareness amongst Muslims to avoid all items containing non-Halal ingredients. This sentiment has now progressed into the field of various medications. It therefore, required a study to assess the knowledge, attitude and perception (KAP) relating to pharmaceuticals containing non-Halal ingredients among doctors working in various hospitals of Malaysia. This was a cross sectional study, carried out in January 2013 - February 2013 period, using a structured, self-administered questionnaires. Study settings included various government hospitals in Malaysia. Data were collected by distributing questionnaires through respective heads of the departments. Study was conducted on a sample of 243 participants. Inclusion criterion was a registered medical doctor working in a government hospital. Descriptive statistics (mean, standard deviation, frequency, percentage, median, inter quartile range) was applied to summarize the data, non-parametric tests were applied. χ^2 Test and Fisher's Exact Test were applied to assess the association between demographic characteristics and knowledge, attitude and perception scores. Results revealed that the hospital doctors had a good and positive attitude and perception about Halal pharmaceuticals. Mean knowledge score out of maximum possible 9 score was 7.67 ± 1.68. Mean attitude score out of maximum possible 45 score was 34.10 ± 5.35 while mean perception score out of maximum possible 55 score was 45.73 ± 5.44. Mean overall KAP score out of maximum possible 109 was 87.60 ± 10.37. There was a significant, positive and weak correlation (0.20 - 0.29) between knowledge and attitude ($r = 0.231$, $p < 0.001$) as well as between knowledge and perception ($r = 0.209$, $p = 0.001$) while there was good correlation (0.5 - 0.75) between attitude and perception ($r = 0.588$, $p < 0.001$). It is concluded from the results that the better knowledge the respondents have on Halal pharmaceuticals the better is their perception and attitude towards Halal pharmaceuticals.

Keywords: knowledge, attitude, perception, KAP, halal, pharmaceuticals

Halal is an Arabic word which means "lawful," "permissible" under Islamic law (1-3). Halal is a universal term that applies to all facets of life; however, this study will adapt this term to refer only to pharmaceutical products that are deemed permissible for consumption of Muslims.

It is a firm belief of all Muslims that Allah is our creator and He is the best judge of what is right for us to consume and in what shape it should be done. However, it is pertinent to mention that all old religions of the world like Hinduism, Judaism and Christianity also command certain religious restrictions and bindings on their followers in the consumption of foods and drinks (4, 5). They may use other terminologies to define these restrictions but the main sentiment is the same. Therefore, it would be pertinent to look into various items of human consumption, including medicines, and their variants, to determine admissibility according to individual beliefs.

The globally expanding Muslim population has in turn expanded the Muslim consumer's market manifold. Moreover, awareness among Muslims is dawning regarding medicines and its sources. Muslim consumers are now increasingly mindful and in search for Halal medicines. Most countries of the world have a body or more to look upon issues related to the Halal food. These bodies govern all matters including issuance of Halal certification. Though, at present, pharmaceuticals are not mandatory for monitoring of the Halal status.

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As a majority of Malaysian population is Muslim (6), there are many government organizations which play active role to ensure provision of Halal foods and pharmaceuticals. Halal Industry Development Corporation (HDC) coordinates the overall development of the Halal industry, and focuses on development of Halal standards, audit and certification (7). Department of Islamic Development Malaysia (JAKIM) has established Malaysia Halal logo and has implemented Halal Certification System (8). Ministry of International Trade and Industry (MITI) promotes Halal products and services (9). Department of Standards, Government of Malaysia has launched world's first standards, MS 2424:2010 (P): for Halal pharmaceuticals. These general guidelines will address the entire pharmaceutical manufacturing and supply chain – from processing to handling, packaging, labeling, distribution, storage and display of medicines and health supplies (10).

Though many government and non government organizations are playing an active role to ensure provision of Halal foods and pharmaceuticals to Muslims in Malaysia, to the best of our knowledge no study has been done, so far, to evaluate the perception of doctors working in hospitals regarding Halal pharmaceuticals. Moreover, their knowledge on the issues surrounding Halal pharmaceuticals is not well explored. Therefore, the main objective of this study is to explore Malaysian hospital doctors' perception and their opinion of Halal pharmaceuticals and to identify barriers to prescribing them.

EXPERIMENTAL

Study settings and design

A cross sectional study design was adopted by using structured, self-administered questionnaire. Survey was conducted across Malaysia from January 2013 to March 2013. Study settings included various government hospitals in Malaysia.

Questionnaire design and validation

After extensive literature review, a self administered questionnaire was designed to conduct this study. The questionnaire was validated by a panel of experts which was composed of eight (8) senior academic researchers and was updated according to their recommendations (11). A pilot study was conducted to evaluate the reliability of the updated questionnaire. Cronbach's α was applied to test validity and internal consistency of the questionnaire (12) and $\alpha = 0.6$ was set as the minimum acceptable value for validity. Final modifications were made based upon the results of pilot study.

The final questionnaire consisted of four parts. The first part of the questionnaire was on respondent's demographic information including age, gender, race, religion, nationality, current position (house officer or specialist), current area of practice, country of basic educational degree, basic medical qualification, post graduate medical qualification and finally the experience. Second part had 9 statements to evaluate the knowledge of respondents. Third part consisted of 11 statements for perception's evaluation and final part had 9 statements about the attitude of respondents regarding Halal pharmaceuticals. All questions were close ended, except one at the end for additional comments.

Scoring method for knowledge, attitude and perception (KAP)

For knowledge statements respondents were asked to choose "yes" or "no" options. Correct answer (yes) was scored one (1) while incorrect answer (no) was scored zero (0). A five point Likert scale was used for perception and attitude statements (strongly agree = 5, agree = 4, neutral = 3, disagree = 2 and strongly disagree = 1). Hence, the minimum and maximum score for knowledge, attitude and perception can be 0 to 9, 1 to 45 and 1 to 55, respectively (11). Total KAP score can be 109.

Selection criteria and recruitment of respondents

All medical doctors working in different departments (except those who were not involved in prescribing i.e., radiologists, pathologists etc.) in selected government hospitals, on duty during the study period and willing to participate in study were recruited as "study participants".

Sampling and data collection

Sampling technique was convenience sampling. All doctors, in selected hospitals, (involved in prescribing medicines) were approached. Only those doctors were included in the study who agreed to participate. Doctors were approached through respective heads of the departments (13) and were requested to return the completed questionnaires within two weeks. Reminders were sent after one week (14). No incentives were offered to any of the respondents (15, 16).

Ethical consideration

Ethical approval to conduct this study was taken from "Joint Ethics Committee of School of Pharmaceutical Sciences, USM and Hospital Lam Wah Ee on Clinical Studies". Additionally, approval of the Ministry of Health Malaysia was also taken.

Table 1. Descriptive characteristics of hospital doctors n = 243.

haracteristics	Demographic characteristics	Frequency (%)
Age (years)	24-28	109 (44.9)
	29-33	71 (29. 2)
	34-40	43 (17.7)
	≥41	20 (8.2)
Gender	Male	102 (42.0)
	Female	141 (58.0)
Race	Malay	90 (37.2)
	Chinese	96 (39.7)
	Indian	8 (19.8)
	Others Punjabi = 4, Mamak = 1, Kadazan = 1, Bisaya = 1	8 (3.3)
Religion	Muslims	93 (38.4)
	Christians	29 (12.0)
	Buddhist	72 (29.8)
	Hindu	40 (16.5)
	Others (Sikh = 5, Tao = 2)	8 (3.3)
Nationality	Malaysian	243 (100)
Current Position	House Officer	64 (26.3)
	Medical Officer	130 (53.5)
	Specialist/consultant	49 (20.3)
Current area of practice	General medicine	103 (42.7)
	Obstetrics and gynecology	38 (15.8)
	Surgery	49 (20.3)
	Pediatrics	28 (11.6)
	ENT	22 (9.1)
	Others	1 (0.4)
Basic medical qualification	MBBS	108 (44.4)
	Doctor in medicine (MD)	124 (51.0)
	Others (MBBCh, BAO = 8, Melo G = 1	11 (4.5)
Postgraduate qualification	Yes	51 (21.2)
	No	189 (77.8)
Country of basic degree	Malaysia	165 (68.5)
	Others (Russia = 20, India = 16, Indonesia = 11, UK = 8, Australia = 5, Ireland = 4, Ukraine = 4, Czech Republic = 2, Canada = 1	76 (31.5)
Working experience (years)	1-4	140 (57.6)
	5-8	51 (21.0)
	9-12	20 (8.2)
	≥ 12	32 (13.2)

Note: The frequencies and percentages are based on observed values; missing values are excluded (18).

Moreover, permission was also sought from directors of respective hospitals and the heads of the departments.

Data analysis

After data collection and screening, data were entered in SPSS version 18. After data cleaning, normality of data was checked by Kolmogorov-Smirnov test. Descriptive statistics (mean, standard deviation, frequency, percentage, median, inter quartile range) was applied to summarize the data. As data were not normally distributed, so non-parametric tests were applied. χ^2 Test and Fisher's Exact Test were applied to assess the association between demographic characteristics and knowledge, attitude and perception score. To find relationship between knowledge-attitude, knowledge-perception and attitude-perception of respondents, Spearman-correlation was applied; p value of 0.05 or less was taken as statistically significant. Responses were considered positive, if they were on positive side of the midpoint of score and negative, if they were on negative side of the midpoint of score (17).

RESULTS

Respondents' demographics

A total of 243 doctors participated in the study. Demographic characteristics of the respondents are depicted in Table 1. Mean age of the respondents was 31.25 years with SD of ± 6.33. Age ranges from 24 to 56 years. Gender - 102 (42%) of the respondents were male and 141 (58%) were female. Ethnicity - 90 (37.2%) were Malay, 96 (39.7%) were Chinese and 48 (19.8%) Indians. According to the religion, 93 (38.4%) were Muslims, 29 (12%) Christians, 72 (29.8%) Buddhists, 40(16.5%) Hindu and 8 (3.3%) from other categories. A total of 64 (26.3%) were house officers, 130 (53.5%) medical officers and 49 (20.3%) were specialists.

Respondents' knowledge regarding Halal pharmaceuticals

Out of maximum possible score (equal to 9), mean knowledge score was 7.67, SD = 1.685. The frequency distribution of respondents' knowledge regarding Halal pharmaceuticals is depicted in Table 2. A total of 42% respondents scored 100% score, 24% respondents scored 88% score while 14% scored 77%. Hence, overall a large majority (> 94%) of the respondents scored 50% and above, which shows that study population has good knowledge regarding Halal pharmaceuticals.

Results showed that all, except one, of the respondents were aware of the term "Halal", while 225 (93%) of the respondents were aware of the term "Haram" ("unlawful", "prohibited", "forbidden") showing a significant association with respect to respondents' religion (p = 0.008) and race (p = 0.003). On the other hand, a total of 181 (74.8%) respondents were aware of the term "Halal pharmaceutical" showing a significant association with

Table 2. Hospital doctors' knowledge about Halal pharmaceuticals (frequeny distribution n = 243).

Statements	Responses	
	Yes n (%)	No n (%)
Are you aware of the term/ word "Halal"?	241 (99.6)	1 (0.4)
Are you aware of the term/word "Haram"?	225 (93.0)	17 (7.0)
Are you aware of the term/word "Halal pharmaceuticals"?	181 (74.8)	61 (25.2)
Do you know that Muslim patients need Halal medicines?	228 (95.0)	12 (5.0)
Do you know that dead animals, blood, pork and Alcohol are Haram for Muslims to use in any form (food, medication etc)?	227 (94.2)	14 (5.8)
Do you know that ingredients of some drugs/medicines are derived from porcine and dead animals?	231 (95.5)	11 (4.5)
Do you know that resources are available to offer Halal alternatives of non-Halal drugs?	173 (72.1)	67 (27.9)
Do you know that it is ethical obligation for a practitioner to take consent from the patient before dispensing any medicine which has any non-Halal content?	200 (82.6)	42 (17.4)
Do you know that most of the doctors are aware of the presence of potentially forbidden animal-derived ingredients in medicines?	183 (75.9)	58 (24.1)

Note: The frequencies and percentages are based on observed values; missing values are excluded (18). The cut off level of ≥ 5 was considered as good knowledge while < 5 as poor.

respect to respondents' gender (Fisher's p value = 0.035), religion (p = 0.005) and years of experience (p = 0.045). A large majority (95%) of the respondents were aware that Muslim patients need Halal medicines showing a significant association with respect to respondents' religion (p < 0.001), race (p = 0.002) and years of experience (p = 0.021).

It was also found that a total of 227 (94.2%) respondents were aware that dead animals, blood, pork and alcohol are Haram for Muslims to use in any form (food, medication etc.), showing a significant association with respect to respondents' religion (p = 0.031) and race (p = 0.016). A majority of respondents (95.5%) were aware that ingredients of some drugs/medicines are derived from porcine and dead animals. This showed a significant association with respect to respondents' race (p = 0.002). A total of 173 respondents (72.1%) had knowledge that resources are available to offer Halal alternatives of non-Halal drugs, showing a significant association with respect to respondents' religion (p = 0.015). Results found that 200 (82.6%) of the respondents had knowledge that it is ethical obligation for a practitioner to take consent from the patient before dispensing any medicine which has any non-Halal content, showing a significant association with respect to respondents' religion (p = 0.003) and race (p = 0.047). Study further found that 183 (75.9%) of the respondents were aware that most of the doctors know of the presence of potentially forbidden animal-derived ingredients in medicines. This showed a significant association with respect to respondents' religion (p = 0.033) and race (p = 0.010).

Respondents' perception regarding Halal pharmaceuticals

The perception of respondents regarding Halal pharmaceuticals was evaluated by using perception questionnaire. The frequency distribution of respondents' perception regarding Halal pharmaceuticals is presented in Table 3. There were total 11 statements to evaluate the perception of respondents. Out of maximum possible score (55), the mean perception score was 45.73 ± 5.44. About 90% of the respon-

Table 3. Hospital doctors' perception about Halal pharmaceuticals (frequency distribution n = 243).

Statements	Responses*				
	SA n (%)	A n (%)	N n (%)	DA n (%)	SDA n (%)
Patient has a right to ask information about sources & ingredients of medicines.	131 (53.9)	107(44)	5 (2.1)		
It is important for prescriber to explain about the sources & ingredients of medicine as much as possible and encourage the patients to ask questions.	78 (32.2)	132 (54.5)	27 (11.2)	4 (1.7)	1 (0.4)
It is not a common practice to inform the patients about sources of the medicines.	21 (8.6)	106 (43.6)	59 (24.3)	45 (18.5)	12 (4.9)
Drug manufacturers should provide prescribers with a list of their products containing animal-derived ingredients.	106 (43.8)	118 (48.8)	16 (6.6)	1 (0.4)	1 (0.4)
Doctor should be educated about the sources of medicines.	96 (39.5)	132 (54.3)	11 (4.5)	2 (0.8)	2 (0.8)
Patient's religious beliefs are considered while prescribing medicines.	99 (41.1)	115 (47.7)	21 (8.7)	5 (2.1)	1 (0.4)
Patient's religious beliefs impact their adherence to drug therapy.	88 (36.2)	122 (50.2)	28 (11.5)	4 (1.6)	1 (0.4)
Pharmaceutical manufacturers should be sensitive towards the requirements of patients and wherever possible should produce Halal medicines.	91 (37.4)	110 (45.3)	37 (15.2)	4 (1.6)	1 (0.4)
Drug companies should clearly mark medication packaging with easy-to-spot Halal/non Halal labels.	102 (42)	109 (44.9)	24 (9.9)	6 (2.5)	2 (0.8)
Clear and well explained guidelines are need of healthcare professionals to navigate religious conflicts.	83 (34.2)	132 (54.3)	26 (107)	1(0.4)	1 (0.4)
Healthcare professionals need to define medical necessity and explore existence of Halal alternatives.	70 (28.8)	133 (54.7)	32 (13.2)	8 (3.3)	

Note: The frequencies and percentages are based on observed values; missing values are excluded [18]. The cut off level of ≥ 28 was considered as positive perception while < 28 as negative. * SA = strongly agree, A = agree, N = neutral, DA = disagree, SDA = strongly disagree.

Table 4. Hospital doctor's attitude about Halal pharmaceuticals (frequency distribution n = 243).

Statements	Responses*				
	SA n (%)	A n (%)	N n (%)	DA n (%)	SDA n (%)
I discuss with patients about forbidden/Haram ingredients of drugs.	41 (16.9)	134 (55.1)	56 (23.0)	11 (4.5)	1 (0.4)
I feel moral obligation to disclose the exact source of non-Halal ingredients to the patient (e.g. alcohol in syrups/elixirs and gelatin in capsules).	55 (22.6)	129 (53.1)	50 (20.6)	7 (2.9)	2 (0.8)
I take consent from patients, if I know the drug is non-Halal.	63 (25.9)	112 (46.1)	53 (21.8)	13 (5.3)	2 (0.8)
I consider patient's religious beliefs when designing a treatment regimen.	71 (29.2)	124 (51.0)	42 (17.3)	6 (2.5)	
I make an effort to search for any available Halal alternatives.	39 (16.1)	103 (42.6)	76 (31.4)	23 (9.5)	1 (0.4)
I educate the patient regarding Halal ingredients.	35 (14.4)	108 (44.4)	80 (32.9)	20 (8.2)	
I prefer Halal medicines in my practice.	59 (24.3)	77 (31.7)	89 (36.6)	15 (6.2)	3(1.2)
I recommend the purchase of Halal alternatives, which may be more expensive.	45 (18.5)	87 (35.8)	88 (36.2)	16 (6.6)	7 (2.9)
I feel that medical representatives are a good source of information about sources and ingredients of drugs for me.	48 (19.8)	114 (47.1)	63 (26.0)	14 (5.8)	3 (1.2)

Note: The frequencies and percentages are based on observed values; missing values are excluded (180). The cut off level of ≥ 23 was considered as positive attitude while < 23 as negative. * SA = strongly agree, A = agree, N = neutral, DA = disagree, SDA = strongly disagree.

dents scored more than 70% of the perception score and all respondents (except one) scored more than 50% of the perception score denoting a positive perception towards Halal pharmaceuticals.

Results showed that a total of 131 (53.9%) respondents strongly agreed and 107 (44%) agreed, that the patient has a right to ask the information about sources of ingredients in medicines, showing a significant association with respect to respondents' religion ($p < 0.001$), race ($p < 0.001$) and current position ($p < 0.001$). A total of 78 (32.2%) of the respondents strongly agreed and 132 (54.5%) agreed, that it is important for prescriber to explain about the sources and ingredients of medicine as much as possible and encourage the patients to ask questions. This showed a significant association with respect to respondents' age ($p = 0.021$), gender ($p = 0.042$), race ($p = 0.031$) and years of experience ($p = 0.025$). On the other hand, 21 (8.6%) respondents strongly agreed and 106 (43.6%) agreed that it is not a common practice to inform the patients about sources of the medicines showing a significant association with respect to respondents' race ($p = 0.014$) and respondents' current area of practice ($p = 0.005$).

It was further found that 106 (43.8%) respondents strongly agreed while 118 (48.8%) agreed that drug manufacturers should provide prescribers with a list of their products containing animal-derived ingredients. This showed a significant association with respect to respondents' race ($p < 0.001$) and religion ($p < 0.001$). A total of 96 (39.5%) respondents strongly agreed while 132 (54.3%) agreed that doctors should be educated about the sources of medicines. This showed a significant association with respect to respondents' race ($p < 0.001$) and religion ($p < 0.001$). A total of 99 (41.1%) respondents strongly agreed and 115 (47.7%) agreed, that patient's religious beliefs are considered while prescribing medicines showing a significant association with respect to respondents' race ($p < 0.001$) and religion ($p < 0.001$). On the other hand, 88 (36.2%) of the respondents strongly agreed while 122 (50.2%) agreed that patient's religious beliefs impact their adherence to drug therapy showing a significant association with respect to respondents' race ($p < 0.001$) and religion ($p = 0.005$).

The study further found that 91 (37.4%) respondents showed their response as 'strongly agree' while 110 (45.3%) as 'agree' that pharmaceutical manufacturers should be sensitive towards the requirements of patients and wherever possible should produce Halal medicines. This showed a significant association with respect to respondents' race ($p < 0.001$), religion ($p < 0.001$) and current position ($p < 0.001$). A total of 102 (42%) respondents showed their response as 'strongly agree' while 109 (44.9%) as 'agree' that drug companies

should clearly mark medication packaging with easy-to-spot Halal/non Halal labels. This showed a significant association with respect to respondents' race ($p < 0.001$), religion ($p < 0.001$) and current position ($p = 0.047$). A total of 83 (34.2%) respondents strongly agreed while132 (54.3%) agreed that clear and well explained guidelines are a need of healthcare professionals to navigate religious conflicts. This showed a significant association with respect to respondents' gender ($p = 0.042$), race ($p < 0.001$) and religion ($p < 0.001$). Furthermore, 70 (28.8%) of the respondents strongly agreed while 133 (54.7%) agreed that healthcare professionals need to define medical necessity and explore existence of Halal alternatives. This showed a significant association with respect to respondents' gender ($p = 0.005$), race ($p < 0.001$), and religion ($p < 0.001$).

Respondents' attitude regarding Halal pharmaceuticals

The attitude of respondents regarding Halal pharmaceuticals was evaluated by using attitude questionnaire. The frequency distribution of respondents' attitude regarding Halal pharmaceuticals is shown in Table 4. There were total 9 statements to evaluate the attitude of respondents. Out of maximum possible score (45), the mean attitude score was 34.10 ± 5.35. More than 77% of the respondents scored 66% of the attitude score while 98% of the respondents scored more than 50% of the attitude score denoting a positive attitude towards Halal pharmaceuticals.

The results showed that 41 (16.9%) respondents strongly agreed while 134 (55.1%) agreed that they discuss with their patients about forbidden/Haram ingredients of drugs, showing a significant association with respect to respondents' current position ($p = 0.002$) and current area of practice ($p = 0.045$). A total of 55 (22.6%) respondents strongly agreed while 129 (53.1%) agreed that they feel moral obligation to disclose the derivation of non-Halal ingredients to the patients (e.g., alcohol in syrups/elixirs and gelatin in capsules). This showed a significant association with respect to respondents' race ($p < 0.001$ and religion ($p = 0.003$). It was further found that 63 (25.9%) of the respondents showed their response as 'strongly agree' while 112 (46.1%) as 'agree' that they take consent from patients, if they know the drug is non-Halal. This showed a significant association with respect to respondents' race ($p = 0.046$) and current position ($p = 0.024$). The study further found that 71 (29.2%) of the respondents showed their response as 'strongly agree' while 124 (51%) as 'agree' that they consider patient's religious beliefs when designing a treatment regimen. This showed a significant association with respect to respondents' race ($p < 0.001$), religion ($p = 0.015$) and current position ($p = 0.013$). A total of 39 (16.1%) respondents showed their response as 'strongly agree' while 103 (42.6%) as 'agree' that they make an effort to search for any available Halal alternatives. This showed a significant association with respect to respondents' race ($p = 0.011$), religion ($p = 0.013$) and current position ($p = 0.005$). A total of 35 (14.4%) respondents showed their response as 'strongly agree' while 108 (44.4%) as 'agree' that they educate the patients regarding Halal ingredients of medicines, showing a significant association with respect to respondents' age ($p = 0.045$), race ($p = 0.002$), religion ($p = 0.011$), years of experience ($p = 0.018$), current position ($p = 0.003$) and current area of practice ($p = 0.001$).

Ir was further found that 59 (24.3%) of the respondents showed their response as 'strongly agree' while 77 (31.7%) as 'agree' that they prefer Halal medicines in their practice, showing a significant association with respect to respondents' gender ($p = 0.003$), race ($p < 0.001$) and religion ($p < 0.001$). A total of 45 (18.5%) respondents showed their response as 'strongly agree' while 87 (35.8%) as 'agree' that they recommend the purchase of Halal alternatives, which may be more expensive, showing a significant association with respect to respondents' race ($p < 0.001$) and religion ($p < 0.001$). It was also found that 48 (19.8%) of the respondents showed their response as 'strongly agree' while 114 (47.1%) as 'agree' that they feel

Table 5. Mean and median score of respondents' knowledge, attitude, perception and KAP about Halal pharmaceuticals.

Variables	Mean ± SD	Median (IQR) (25-75)
Knowledge	7.67 ± 1.68	8 (7-9)
Attitude	34.10 ± 5.35	34 (31-36)
Perception	45.73 ± 5.44	45 (43-50)
KAP	87.60 ± 10.37	88 (81-95)

Table 6. Correlations between knowledge, attitude and perception*.

Variables	Number. of respondents (n)	p value	Correlation (r)
Knowledge-Perception	243	0.001	0.209
Knowledge-Attitude	243	< 0.001	0.231
Attitude-Perception	243	< 0.001	0.588

*Correlation significant at 0.01 levels (2 tailed).

that medical representatives are a good source of information about sources and ingredients of drugs for them showing a significant association with respect to respondents' race ($p < 0.001$) and religion ($p < 0.001$).

Correlation between knowledge, attitude and perception

Correlations between knowledge, attitude and perception are depicted in Table 6. There was a significant, positive, and weak correlation (0.20 - 0.29) between knowledge and attitude ($r = 0.231$, $p < 0.001$) as well as knowledge and perception ($r = 0.209$, $p = 0.001$), whereas good correlation (0.5 - 0.75) between attitude and perception ($r = 0.588$, $p < 0.001$). This means that the better knowledge the respondents have on Halal pharmaceuticals, the better is their perception towards them.

DISCUSSION AND CONCLUSION

This study was conducted to evaluate the knowledge, attitude and perception of doctors working in various government hospitals of Malaysia. A total of 243 doctors participated in the survey. Extensive literature review found only one study which is conducted regarding Halal pharmaceuticals among doctors working in various hospitals (11). Medicines has become a necessity now to maintain health. Usually there are three players in this context: physicians, pharmacists and consumers (19). Consumers usually cannot judge which medicine is suitable for them. This is then the role of physician to choose the most suitable medication for his/her patient keeping in mind the religious beliefs of the patient as well. Consumers who are the end users of the medicines are a key success factor for the treatment process. Therefore, any lack of knowledge, misconception or mal practice regarding medicines would negatively affect on drugs utilization, patients' quality of life and country resources. This is an accepted and undeniable finding that physicians are perceived as one of the most knowledgeable healthcare practitioners on drugs and medications being used. In fact, closer patient-physician relationship has resulted in consumer to perceive that the advice of physician is reliable. Hence, physician's opinion becomes an important factor when it comes to the drug decision making process. An important aspect of consideration when prescribing a medication regimen is the patient him/herself. Individuals have different views on treatment, including the use of certain inactive ingredients in medications. However, most patients are unaware of these ingredients in their medications. The clinicians and pharmacists should be proactive and not leave it to the patient to broach the subject. Since patients have the right to make informed decisions about their medical treatment, it is important that healthcare providers involve the patient when making treatment decisions (20).

In this study we tried to explore the knowledge of doctors working in hospitals about Halal pharmaceuticals. Study findings showed that doctors had a good knowledge towards issues surrounding Halal pharmaceuticals. More than 94% of the respondents scored more than 50% of the knowledge score. Significant association was found between gender, race, religion, years of experience and different statements of knowledge.

This study found positive perception about Halal pharmaceuticals. Almost all of the respondents scored more than 50% of the perception score. A majority of the respondents perceived that "patients have a right to ask information about sources of ingredients in medicines which are prescribed to them". A large majority of the respondents agreed that 'drug companies should clearly mark medication packaging with 'Halal' or 'non Halal' logo. This approach is also described by Khokhar et al. while discussing faith issues in psychopharmacological prescribing (21). This would be a novel and convenient approach, if drug manufacturers practice to mark drugs clearly about Halal or non-Halal, then it will be easy for a medical practitioner to make a better choice for the patients. A majority of respondents perceived that doctors should be educated more about Halalness of medi-

cines; moreover, doctors should inform their patients about Haram ingredients. This is in accordance with Newson's "Clinical Ethics Committee Case Report" in which this issue is highlighted (22). A large majority of respondents perceived that patient's religious beliefs impact their adherence to drug therapy. This is in line with Sattar et al. (23), who reported four different cases of patient's non-adherence due to religious beliefs. A majority of respondents either strongly agreed or agreed that pharmaceutical manufacturers should be sensitive towards the requirements of Muslim patients and wherever possible should produce Halal medicines. This is in accordance with what is reported by Bashir et al. (24) while discussing "Concordance in Muslim patients in primary care". A large majority of respondents perceived that drug manufacturers should provide prescribers with a list of their products containing animal-derived ingredients to assist the prescriber. This approach is in line with what is reported by Hoesli and Smith while discussing effects of religious and personal beliefs on medication regimen design (20). Significant association was found between age, gender, race, religion, years of experience, current position (medical officer, house officer or specialist), current area of practice and different statements of perception.

Study respondents have positive attitude towards Halal pharmaceuticals. A large majority of the respondents scored 50% or more of the attitude score. A large majority recommended purchasing Halal medicines, taking consent from their patients, educating the patients about Halal ingredients of medicine and discussing with their patients about Haram ingredients of medicines. Significant association was found between age, gender, race, religion, years of experience, current position (medical officer, house officer or specialist), current area of practice and different statements of attitude.

A significant, positive correlation was found between knowledge and attitude, attitude and perception as well as knowledge and perception. This means that the better knowledge the respondents have on Halal pharmaceuticals, the better their perception will be towards them. A majority of respondents perceived that patient's religious beliefs should be considered while doctors decide medication for them.

Within this context, this research shows that religion and spirituality are linked to positive physical and mental health. A study from the University of Missouri, Columbia shows that if religious issues are addressed during treatment, even individuals with disabilities can adjust to their impairments and it can give a new meaning to their lives (25). Therefore, it is advisable to disseminate adequate information about Halal medicines in the Malaysian population. This dissemination of information will result in building positive perception towards medications and an increased treatment outcome, which is the aim of any therapy.

Summarizing the issue related to Halal pharmaceuticals, it can be said that healthcare continues to mature. Previously, patient care was largely thought to involve simply the correct application of medical science to disease. Today, however, the slogan is that "an ounce of prevention" is often better than a "pound of intervention." Religious issues do carry their weight and importance to health outcomes. However, a significant question in providing the best quality of health care is "how can we offer our patients, with their rich diversity of religious backgrounds, care that is spiritually nurturing and culturally competent?" Addressing concern associated with Halal and Haram in medication use can be the first step in providing competent and rational healthcare.

To summarize this discussion, we can say that this study is an indicator that the knowledge, attitude and perception regarding Halal/Haram status of medicines, among doctors working in hospitals, were good where 94%, 98%, and all (except one) of the respondents scored 50% and above, respectively. Significant correlations were found between knowledge and attitude, attitude and perception as well as knowledge and perception.

Suggestions for further study

This issue is of paramount importance for Muslims as it affects their religious beliefs directly and should be researched and explored in various parts of, not only Malaysia but, the entire Muslim world so that more pertinent results come to the focus of the various players in the field of pharmaceuticals.

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In the paper: „NEW RENIN INHIBITORS CONTAINING PHENYLALANYLHISTIDYL-γ-AMINO ACID DERIVATIVES IN P3- P1 POSITION" Vol. 71 (1), pp. 59-69 (2014) the following text was omitted:

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In the paper: "FULLERENES AS THE CARRIERS OF COMPOUNDS WITH AMIDE BOND" Vol. 71 (6), pp. 1073-1078 (2014) and in the respective page of contents of this issue, the following authorship should be read:

MONIKA K. GRUDZIEŃ[1]*, MARCIN SUSKIEWICZ[1], EDITA AJMANOVIČ[1], MARZENA MĘDREK[1], FRANCISZEK A. PLUCIŃSKI[2] and ALEKSANDER P. MAZUREK[1,2]

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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).
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