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**REVIEW**

# PHARMACOLOGY AND SYNTHESIS OF DAURICHROMENIC ACID AS A POTENT ANTI-HIV AGENT

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**Abstract:** Daurichromenic acid (a potent anti-HIV agent) has been surveyed in this review article, not only for its pharmacological assessment comparative to other compounds but also for methodological trends in different synthetic approaches.

**Keywords:** anti-HIV, 2H-chromen, total synthesis

Acquired Immunity Deficiency Syndrome (AIDS), as is evident by the name, is a disease related to immune system of the human body. It was first reported in the United States in 1981. On acquiring this syndrome the infected human underwent collapse of the immune system, opportunistic infections and cancers. AIDS is caused by a retro virus known as HTLV-III or LAV and it belongs to the family *Lentiviridae*. It is also commonly known as Human Immunodeficiency Virus (HIV). This virus has the tendency to invade the central nervous system (CNS) where it can cause neurological destruction (1).

Reverse transcriptase enzyme in the human body is utilized by HIV to replicate itself. Zidovudine, which is a thymidine analogue, was the first anti-HIV drug, tested in 1984-1985 and was found effective against HIV in rodents and *in vitro* (2-6). There were some side effects too associated with the long term dosage of zidovudine. These include anemia, neutropenia, hepatotoxicity, cardiomyopathy and myopathy. It had been found that these side effects were caused due to high dosage use in the early trials and could be controlled by reducing the drug dose. Some of the origins of these side effects were found to be the depletion of thymidine triphosphate, possible oxidative stress and depletion of intracellular L-carnitine or apoptosis of the muscle cells. The transient depletion of mitochondrial DNA and the sensitivity of an enzyme (γ-DNA polymerase) in the mitochondria of some cells were also found to be the possible reasons of the side effects of zidovudine (7, 8). It has been found that the erythropoietin hormone enhances the production of red blood cells (RBCs) and can in turn control anemia caused by zidovudine (9, 10). The therapeutic strength of zidovudine has been found to be increased by the use of medicines (such as aspirin, nordazepam etc.) which can reduce or inhibit the hepatic glucuronidation. Occasional reports of side effects include mood swings as well as discoloration of skin and nails. Common is the acid reflux, weakness, breathing problems, headache, abdominal fat reduction and increased heartbeat. Above all, this medicine has been designated as possible carcinogenic (11). The dilemma is that the potency of zidovudine is not good enough to stop the replication of HIV altogether. It results in the mutation of

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reverse transcriptase and development of resistant strains of HIV occurs (12).

Another reverse trasnscriptase inhibitor is zalcitabine (13). It is pyrimidine derivative. It was marketed in 1992 as single therapy. This drug was found to be relatively less potent; therefore, it was again marketed in 1996 as a combination drug with zidovudine (14). Unfortunately, this medicine is associated with serious side effects. It has been found that the treatment of patients having advanced HIV infections with combination of zidovudine and zalcitabine is not superior to zidovudine alone (15). These are the reasons the medicine has been discontinued since 2006. This medicine should not be administered with those which can inhibit the phosphorylation process (such as lamivudine) or can cause peripheral neuropathy (such as didanosine). Ulcer (16), nausea and headache are some other side effects of this medicine.

Another medicine used for inhibiting reverse transcriptase of HIV is stavudine. It is also thymidine analogue. Peripheral neuropathy is one of the adverse effects of stavudine. Like zidovudine, decreasing the dose of stavudine can overcome this side effect. The *in vitro* testing shows the tendency of the medicine towards disrupting the genetic information whereas clinical trials do not point out any carcinogenicity. Another side effect of this medicine is that it causes degeneration of adipose tissue (17). For this reason, it is not in use these days in most of the countries for HIV treatment. Although, it has been discontinued in most of the countries since 2009, due to its low price, it is still in use in the developing countries as a treatment of AIDS (18).

It has been observed that all the anti-HIV agents have the tendency to be lipophilic (1). For example, zidovudine has azido group. This group helps the medicine to cross membranes and blood-brain barrier by diffusion. The mode of action of zidovudine is to selectively inhibit the reverse transcriptase enzyme of HIV that is needed to make a DNA copy of its RNA. The double stranded DNA produced as a result gets incorporated into the host cell DNA. The active form of zidovudine is 5´-triphosphate which discontinues the formation of DNA chain of HIV. If administered in high dose, the medicine can also inhibit DNA polymerase which is used by the healthy cells of human body to replicate. Selectivity comes from the fact that the human DNA has the tendency to repair the broken strands whereas HIV's DNA does not have this ability (19). The lipophilicity trend can be also seen in zalcitabine. This drug also gets phosphorylated in the cells and converts itself into the active triphosphate form. This active form selectively discontinues the production of DNA of HIV by providing itself as a substrate for HIV reverse transcriptase thereby halting the replication process due to the unavailability of hydroxyl group. The medicine has half-life of two hours, can be administered orally, renal elimination occurs (20). The activity of stavudine is also dependent upon its activated triphosphate form within the cell by cell kinases. This medicine also presents itself as a natural substrate and stops the replication of HIV DNA by getting incorporated into the DNA strand (21). The property of zidovudine to inhibit the phosphorylation of stavudine within the cell makes it less suitable to administer both together. Stavudine can be administered orally; it gets excreted *via* urinary as well as endogenic pathways (22).

The $IC_{50}$ value (the concentration required to produce 50% decrease in supernatant reverse transcriptase) for zidovudine was 0.013 µg/mL in both HIV-infected H9 cells and peripheral blood lymphocytes (23); $IC_{50}$ for zalcitabine is 0.338 µg/mL (24) and for stavudine is up to 0.8968 µg/mL (25).

The development of resistant strains against these medicines is not a healthy sign. The need of the hour is to find out some more lead compounds, which must be potent enough to deal with the HIV. Many scientists look towards Mother Nature to seek cures for lethal diseases. History shows that there are many examples where scientists found remedy directly or in modified form from natural products. Most commonly observed structural units in natural products are six membered oxygenated heterocycles (pyranes). Daurichromenic acid is an example, where oxygenated ring is present in the form of 2*H*-1-benzopyran (also known as 2*H*-chromen). This moiety is an important structural unit in different members of almost every class of naturally occurring phenolic compounds such as flavonoids, coumarins, rotenoids, stilbenoids and chromene glucosides. A number of pharmacological active compounds possess this 2*H*-chromen ring system in their structure. These compounds are not only important sources for the synthesis of other natural products (26-31) but also act as anti-depressant, anti-hypertensive, anti-ischemic, anti-fungal, anti-tumor, active against snake venom and most importantly anti-HIV agents (27).

Daurichromenic acid has been reported as an effective anti-HIV agent with 0.00567 µg/mL $EC_{50}$ value, when tested against severely infected H9 cells. In the same study, daurichromenic acid has been tested for its inhibitory activity towards uninfected H9 cells, and has showed $IC_{50}$ value of 21.1

µg/mL. An overall therapeutic index (TI) of daurichromenic acid has been rated good with TI value of 3710. Two isomers of daurichromenic acid have also been tested for anti HIV activity, where rhododaurichromanic acid A exhibited comparatively potent anti-HIV activity with $EC_{50}$ value of 0.37 µg/mL and TI value of 91.9, but rhododaurichromanic acid B has not shown any anti HIV activity (27, 28, 32-42).

**Comparison of daurichromenic acid with other 2*H*-chromen based compounds**

A study has been carried out on eight different compounds having 2*H*-chromen structure. Out of these eight compounds, (+)-calanolide A **1** and (-)-calanolide B **2** (Fig. 1) (43) displayed potency against HIV replication in human T-lymphoblastic (CEM-SS) cells. The $EC_{50}$ and $IC_{50}$ values for **1** were 2.7 µM (1.00019 µg/mL) and 13.0 µM (4.81572 µg/mL), respectively. It was evaluated that **2** also had almost equal potency against HIV when compared with **1**. However, the enantiomers of both **1** and **2** were inactive against HIV (27). On the other hand, the potency of daurichromenic acid, with $EC_{50}$ value of 0.00567 µg/mL, is about 176 times higher than these calanolide molecules. This represents the importance of daurichromenic acid compared to other 2*H*-chromens when it comes to potency against HIV.

A study on the structure-activity relationship were conducted on A **1** and B **2**. In this study 14 different analogues of **1** and **2** were tested on human lymphoblastoid CEM-SS cells in the NCI primary anti-HIV screening assay. The results obtained from these analogues, when compared with the results obtained for both **1** and **2**, showed that a heteroatom is necessary for the anti-HIV activity of these compounds. The relative potency of ketone and azide clearly indicates that a hydrogen bond acceptor is required at position 12. Moreover, the potency of these compounds decreases with increasing bulk of substitution at position 12, for instance (=O > $N_3$ > OAc). This clearly demonstrates that there is either special limitation or stereoelectronic requirement at

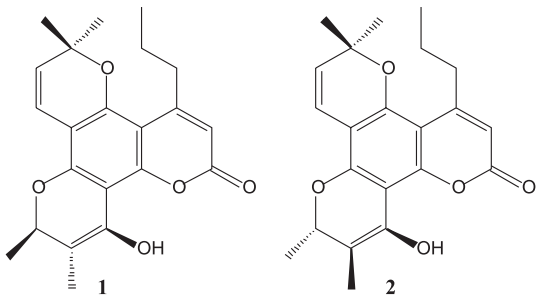


Figure 1. (+)-calanolide A **1** and (-)-calanolide B **2**

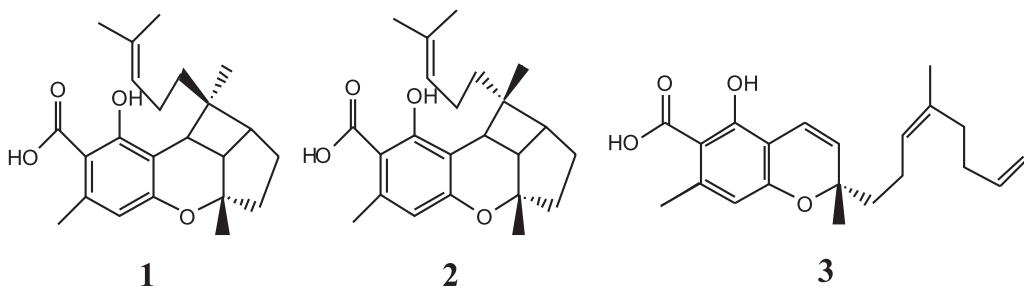


Figure 2. Rhododaurichromanic acid A **1**, rhododaurichromanic acid B **2** and daurichromenic acid **3**

position 12. The oxygen substituent at position 12 must possess "S" configuration. Therefore, 12β-hydroxy group is important for anti-HIV activity (27, 44).

**Comparison of (+)-daurichromenic acid with grifolin, grifolic acid and grifolic acid methylester**

Synthesis of daurichromenic acid from grifolia has been reported (38). For the estimation of anti-HIV behavior of starting materials, grifolin, grifolic acid and its methylester were tested for anti-HIV effects. These compounds possess anti-HIV activity, which has been categorized as follows:

grifolin > grifolic acid > grifolic acid methylester

$EC_{50}$ values of these compounds were compared with that of (+)-daurichromenic acid. The comparison showed that anti-HIV potential of (+)-daurichromenic acid was about 6700 times better than grifolin, 7000 times better than grifolic acid and 9000 times better than grifolic acid methylester (38).

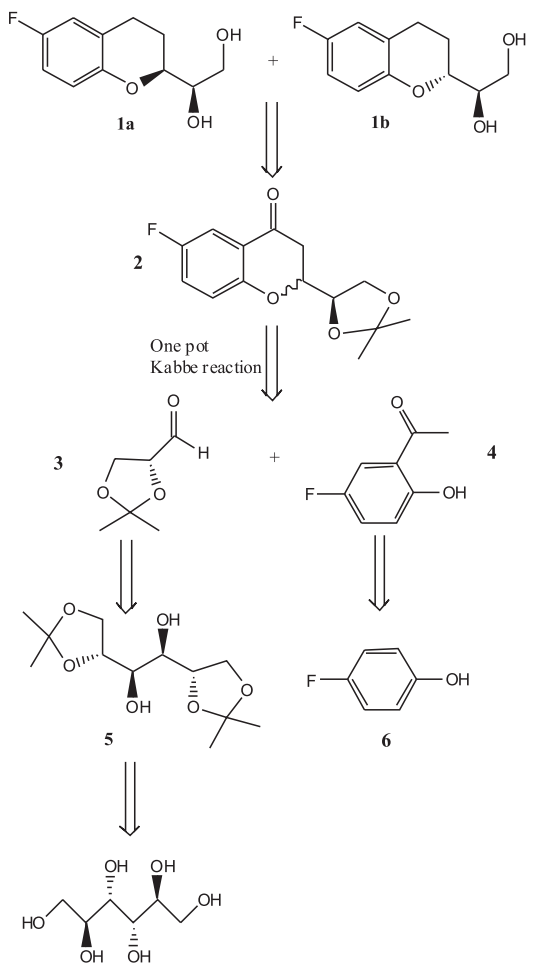


Figure 3. Kabbe reaction in chromen synthesis

**Isolation of compound and its derivatives**

*Rhododendron dauricum* (Ericaceae) is a Chinese medicinal plant which is widely distributed in Asia. Daurichromenic acid **3** along with its chromane derivatives rhododaurichromanic acid A **1** and B **2** (Fig. 2) had been isolated from the leaves and twigs of this plant during its screening for novel anti-HIV agents. In the first instance, the methanol extract of the plant showed promising anti-HIV activity (27, 28, 32-37, 39, 42, 45-48). The $EC_{50}$ was less than 20 µg/mL with a TI value less than 5 (27, 39). In the next step, partitioning of methanolic extract was done between ethyl acetate and water. The anti-HIV potential was tracked in the ethyl acetate fraction. This fraction was then further partitioned between *n*-hexane (non-polar) and 90% methanol (polar) fractions. The bioassay guided study revealed that the *n*-hexane fraction was possessing anti-HIV activity. This potent fraction was then subjected to a multifractionation approach; first by silica gel and next by semi preparative HPLC. This bioassay guided fractionation resulted into **1**, **2** and **3**. The absolute configuration of carbon number 2 in **3** is 'S' (28, 34, 35, 39, 47).

In 2009, **3** was isolated from the leaves of another plant known as *Rhododendron adamsii* (41). The benzene extract of the plant was subjected to reverse phase chromatography and eluted with aqueous solution of methanol in an increasing percentage. The last fraction obtained by pure methanol elution was dried and chromatographed using silica gel as stationary phase and ethanol/chloroform as eluent mixture with stepwise increasing percentage of ethanol. Although, a new source of **3** has been discovered, the total percentage of **3** in the extract was only 0.1% (41).

In the year 2010, **3** was isolated from another plant known as *Rhododendron anthopogonoides*. This plant was collected from Sichuan, China in the year 2003. In this study, the plant leaves and twigs were extracted with 60% ethanol. The extract was fractionated into four fractions; *n*-hexane, water, butanol and ethyl acetate. Normal and reverse phase chromatography as well as normal phase HPLC of *n*-hexane fraction resulted in six compounds one of which was **3** (49).

**Total synthesis of daurichromenic acid**

Daurichromenic acid can be synthesized by uniting benzene ring derivative **4** with pyrone ring

moiety **3**. For this purpose a study has been conducted to design the route for efficient synthesis of chiral chromane intermediates. The retrosynthetic analysis is shown in Figure 3 (50). As per adopted scheme, Kabbe reaction was employed to synthesize pyranone skeleton **2**, which was then converted to chromanone **1a** and **1b**. Then, reduction of **1a** and **1b** resulted in chroman (50), and subsequently in chromen on the route to synthesize daurichromenic acid.

Structural elucidation and stereochemistry of daurichromenic acid cannot be determined through straightforward instrumental analyses, whereas, its chromane derivative, rhododaurichromanic acid A can be analyzed through X-ray crystallography. An indirect structure elucidation and absolute stereochemistry assessment of daurichromenic acid has been reported through its photochemical conversion into rhododaurichromanic acid A and rhododaurichromanic acid B. Isomerization of *trans* double bond at C11-C12 in daurichromenic acid has been reported before photochemical cyclization to produce rhododaurichromanic acid B (32).

Total synthesis of methyl (±)-daurichromenic ester **5** and (±)-rhododaurichromanic acid A and B **'6'** and **'7'** reported by another group (27) has been shown in Figure 4. In this exertion, *trans,trans*-farnesal **1** was reacted with symmetrical 1,3-cyclohexanedione **2** in the presence of piperidine. Reaction conditions favored condensation and electrocyclization of piperidine with **1**, which resulted in the production of 2*H*-pyran ring structure **3** (yield = 70%). Compound **3** was converted to methyl ester **4** in the presence of lithium diisopropyl amide and N≡C-COOMe (yield = 71%). Then, dehydrogenation of **4** was carried out in the presence of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) yielding **5** (yield = 44%). A hydrolysis reaction of **5** was

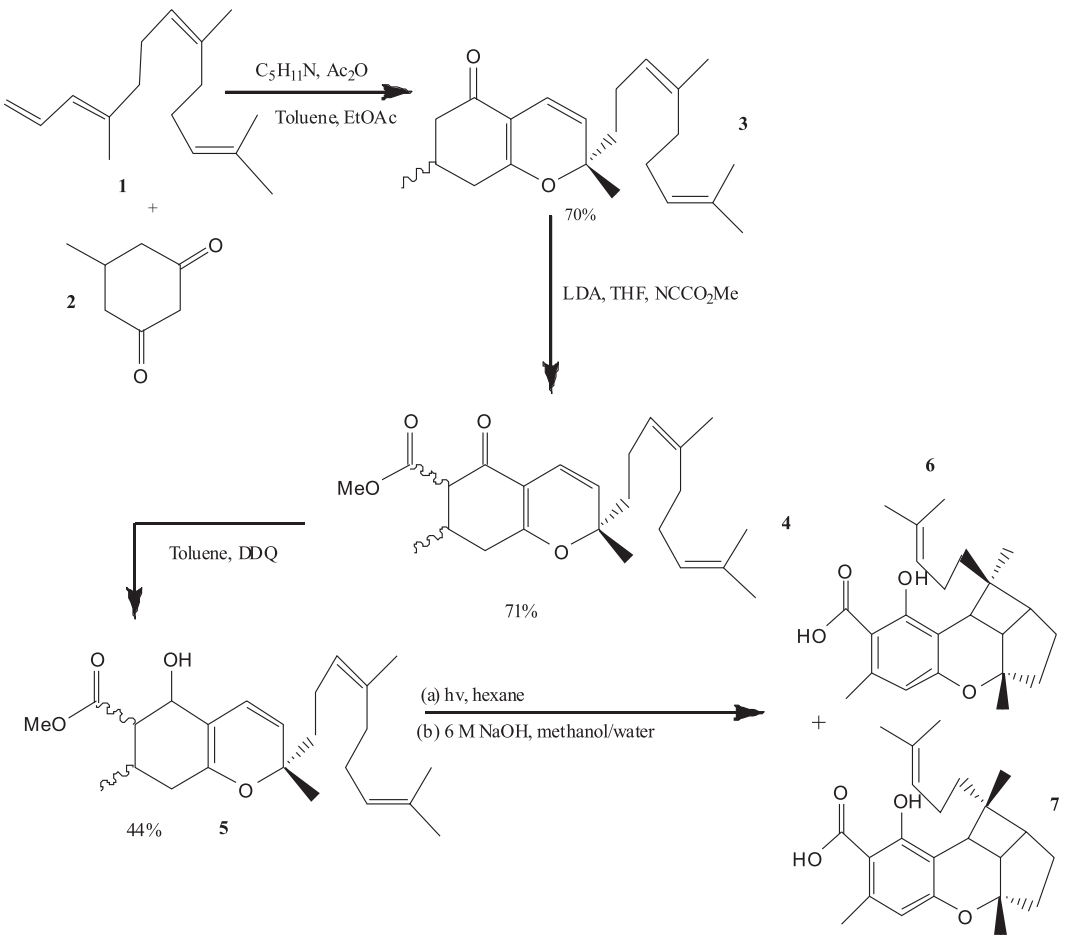


Figure 4. Synthesis of daurichromenic ester **5** and (±)-rhododaurichromanic acid A and B **6** and **7**. DDQ = 2,3-dichloro-5,6-dicyanobenzoquinone

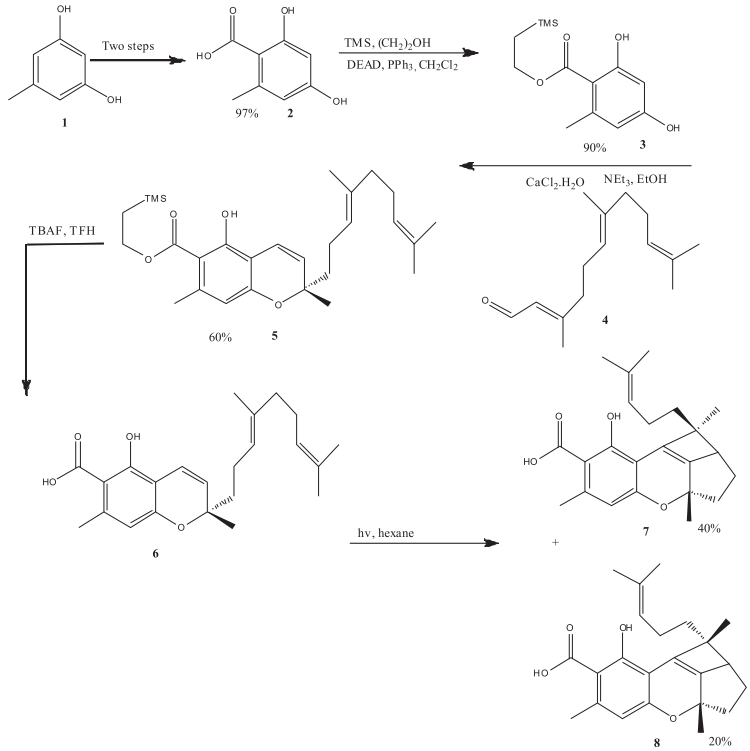


Figure 5. Synthesis of (±)-daurichromenic acid **6**, (±)-rhododaurichromanic acid A **7** and B **8** by using orcinol **1**

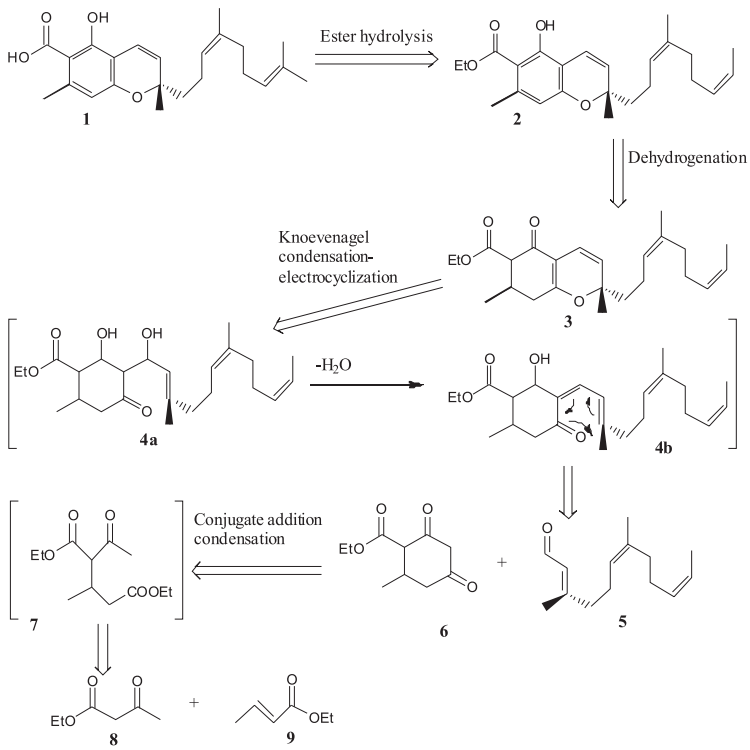


Figure 6. Retrosynthetic analysis of (±)-daurichromenic acid

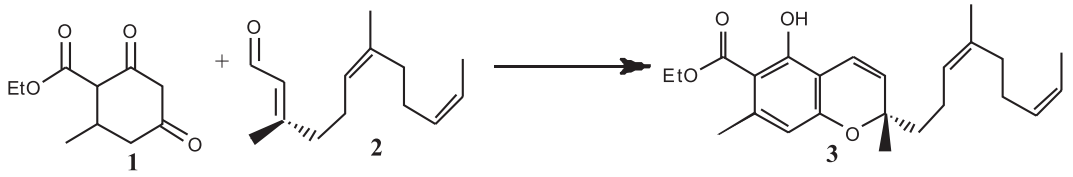


Figure 7. (±) Daurichromenic acid synthesis with Knoevenagel condensation and electrocyclization

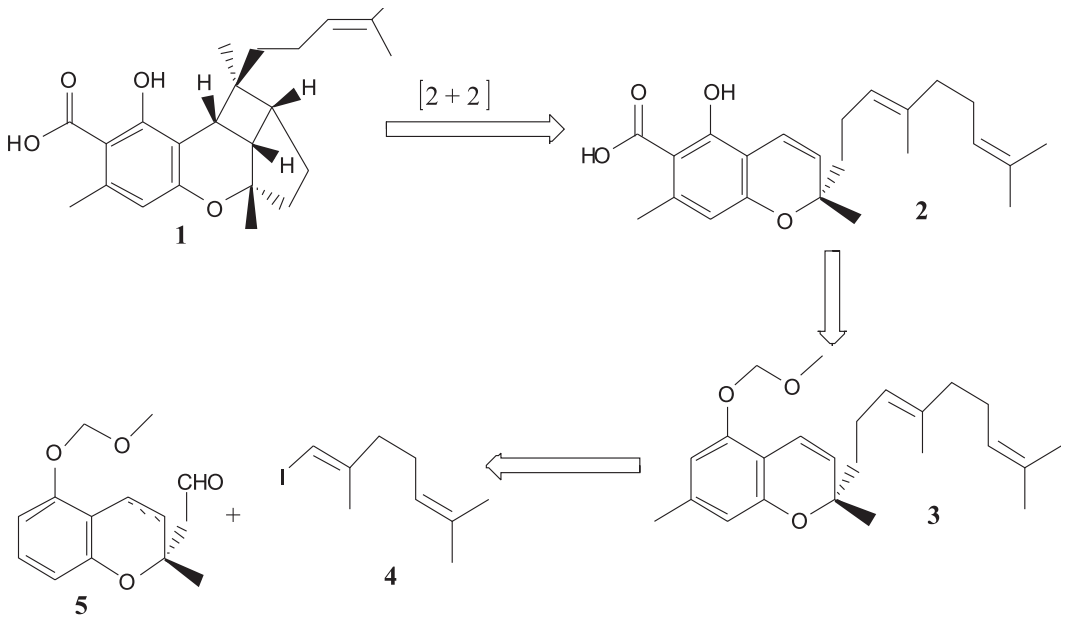


Figure 8. Retrosynthesis of rhododaurichromanic acid A **1** and daurichromenic acid **2**

planned to obtain (±)-daurichromenic acid but Hsung and coworkers could not establish the suitable reaction conditions for this purpose. So, **5** was first photochemically cyclized and then saponified to yield (1 : 1) mixture of **6** and **7** (yield = 74%) (28, 32, 34, 45-47).

In the same time, another group published quite efficient and concise scheme for total synthesis of (±)-daurichromenic acid **6** shown in Figure 5. In this scheme, orcinol **1** was converted into β-trimethylsilyl ethyl ether **3**. Microwave energy has assisted in condensation of **3** with *trans,trans*-farnesal **4** and yielded an ester **5** (yield = 60%). Deprotection of **5** in the presence of THF and tetra-*n*-butyl ammonium fluoride yielded **6** (yield = 94%) (32, 46, 51, 52). A photochemical conversion of **6** (in hexane) produced (±)-rhododaurichromanic acid A **7** (yield 40%) and B **8** (yield = 20%) (28, 32, 34, 45, 47). Meanwhile, another attempt was carried out to synthesize (±)-daurichromenic acid **1** (27). The retrosynthetic approach of this synthesis is presented in Figure 6.

According to this approach, conjugate addition and intramolecular condensation reaction between α,β-unsaturated esters **9** and alkyl acetoacetates **8** may result in 1,3-cyclohexanedione precursors **6**. The Knoevenagel condensation of unsymmetrical **6** with α,β-unsaturated aldehydes **5** would produce β-hydroxycarbonyl intermediates **4b** (the reactions of compounds having carbonyl functionality with active methylenes, for example, malonates and acetoacetates, follows the mechanism of Knoevenagel condensation. The catalytic amount of a base (amine) facilitates the production of alkylidene- or benzylidene-dicarbonyl compounds). Compound **4b** on dehydration converts into **4a** and followed by electrocyclization would yield 2*H*-pyrans **3**. Furthermore, **3** on dehydrogenation (oxidation/aromatization) results into **2** and ester hydrolysis of **2** can yield **1** and its analogues (27, 32, 46).

Based on retrosynthetic scheme, two different analogues of cyclohexanediones have been prepared either having a methyl or a phenyl substitution. Methyl substitutd species (yield = 81%) have been reported from ethyl acetoacetate and ethyl crotonate, whereas phenyl substituted compound (yield = 61%) has been reported from methyl acetoacetate and methyl cinnamate. These methyl and phenyl moieties verified ease of substitution at position 7 in daurichromenic acid. However, according to this study, such substitutions at position 5 and/or 6 were not possible by using methyl acetoacetate and analogous commercially available α,β-unsaturated methyl esters by this direct procedure. Commercially available α,β-unsaturated aldehyde "3,7-dimethyl-2,6-octadienal" (Citral, E/Z ≅ 2 : 1) has been utilized for the synthesis of (±)-daurichromenic acid. Condensation of methyl substituted cyclohexanedione with citral has yielded the precursor of (±)-daurichromenic acid (yield = 87%). The condensation was carried out at room temperature for about 3 h, in the presence of 5 mol % of 1,2-ethylenediammonium diacetate. This thermal treatment of prepared ester with DDQ in benzene for 4-16 h converted the carbonyl group at position 5 to hydroxyl group and yielded the desired precursor of (±)-daurichromenic acid (yield = 11%). The saponification of this precursor with an aqueous solution (5 M) of sodium hydroxide (~10 equiv.) in dimethyl sulfoxide on heating at 80°C for ~16 h yielded (±)-

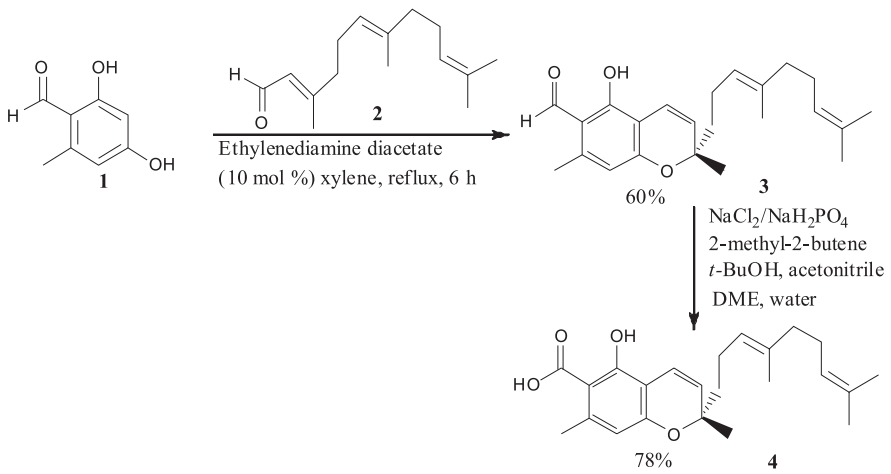


Figure 9. Synthesis of daurichromenic acid **4** from 2,4-dihydroxy-6-methylbenzaldehyde **1**

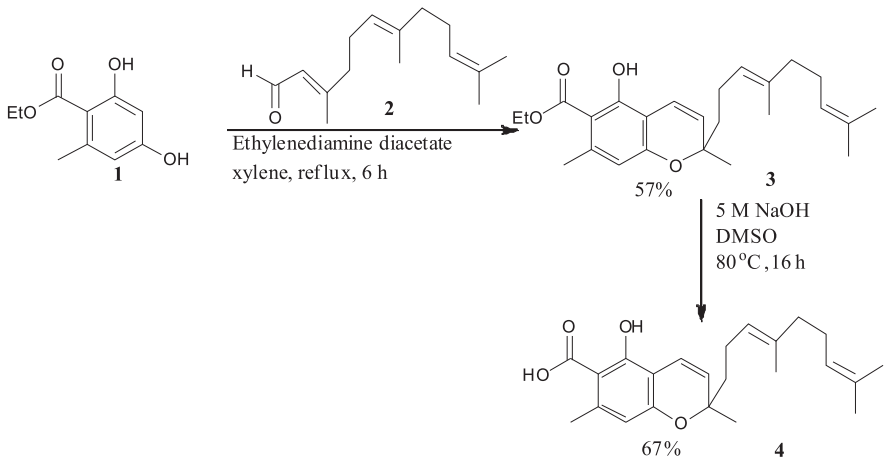


Figure 10. Synthesis of daurichromenic acid **4** from 2,4-dihydroxy-6-methylbenzoate **1**

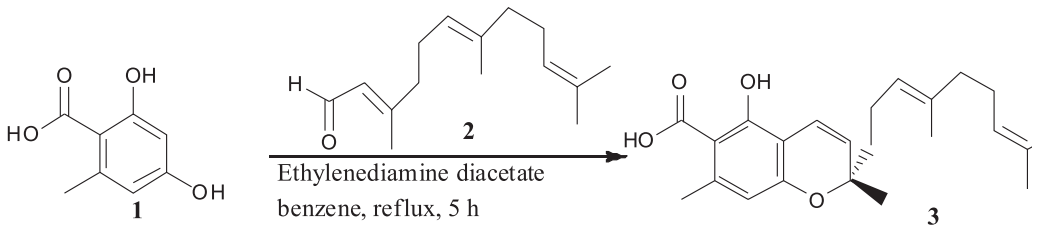


Figure 11. Synthesis of daurichromenic acid **3** from 2,4-dihydroxy-6-methylbenzoic acid **1**

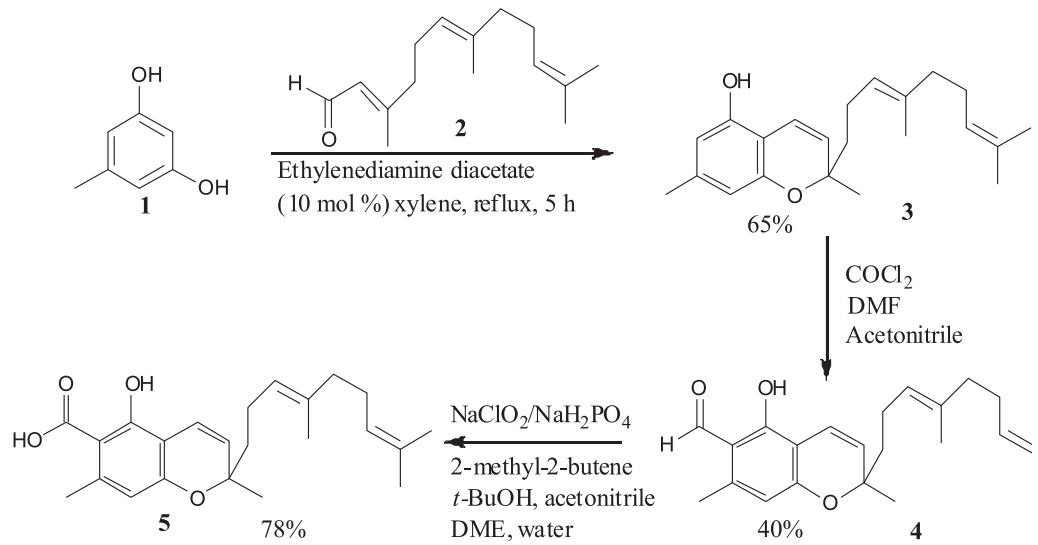


Figure 12. Synthesis of daurichromenic acid **5** from orcinol **1**

daurichromenic acid with a yield of 76%. The spectroscopic data for the synthesized (±)-daurichromenic acid were in full agreement with these reported in the literature for naturally isolated (±)-daurichromenic acid (32). A number of other daurichromenic acid analogues had also been prepared in this study, which signifies that a diverse substituents can be introduced at position 2 and position 7 in the analogues of daurichromenic acid (32). The retrosynthetic approach of another synthesis has been presented in Figure 8 (42).

According to this approach, the daurichromenic acid **2** and rhododaurichromnic acid A **1**, both, can be synthesized by making a carbon-carbon bond between the chiral chromane **5** and vinyl iodide **4** (42). The efforts made to synthesize **1** established that the palladium catalyzed asymmetric allylic alkylation reaction of phenol allyl carbonates facilitate the formation of chiral chromanes and further help in their oxidation to yield chiral chromens. The introduction of the side chain was completed by regioselective allylic deoxygenation using palladium as a catalyst (42).

In the year 2005, another attempt was made to synthesize daurichromenic acid by using either 2,4-dihydroxy-6-methylbenzaldehyde **1** or 2,4-dihydroxy-6-methylbenzoic acid as shown in Figure 9. Both of these starting materials are commercially available thereby making the synthetic route relatively simpler than before. Moreover, the use of **1** as a starting material eliminates the hydrolysis step in the daurichromenic acid **4** synthesis (45). Refluxing of **1** with *trans,trans*-farnesal **2** in xylene for 6 h was carried out in the presence of catalytic amounts of ethylenediamine diacetate (10 mol %). The yield of adduct formed was 60%. The resulting 2*H*-benzopyran **3** in the presence of buffered sodium chlorite at room temperature yielded **4** after 10 h (yield = 78%) (45).

The same group of scientists modified the above mentioned reaction conditions in another

study. They replaced the aldehyde with ethyl 2,4-dihydroxy-6-methylbenzoate **1**, as shown in Figure 10, and refluxed it with **2** in the presence of ethylenediamine diacetate (20 mol %). Although the reaction was successful, the yield of resultant **3** was reduced to 57%. For hydrolysis, 5 M sodium hydroxide was used this time; temperature was raised to 80°C and time was increased to 16 h. Compound **4** produced as a result was significantly lower in yield than before (yield = 67%). The spectroscopic data of **4** synthesized was in agreement with the one published (47, 53).

In a quest to further improve the synthesis of **4** and to bring it to one step synthesis, 2,4-dihydroxy-6-methylbenzoic acid **1** was refluxed with **2** in the presence of ethylenediamine diacetate (10 mol%) in benzene for 5 h. Compound **4** was produced in just one step (yield = 59%) (28).

From the studies of this research group, it can be concluded that shuffling between aldehyde, ester and acid as a starting material or changing the aromatic solvent can affect the yield of the daurichromenic acid produced. Although the best yield of daurichromenic acid obtained was 78% by using 2,4-dihydroxy-6-methylbenzaldehyde as a starting material but the more promising scheme is the one step synthesis of daurichromenic acid with 2,4-dihydroxy-6-methylbenzoic acid as a starting material and benzene as a solvent. It should be noted that the yield of daurichromenic acid in case of one step synthesis is 19% less than the maximum reported yield, this one step synthesis is cost effective, as it requires less chemicals.

When these researchers replaced benzoic acid, aldehyde or ester as a starting material all together with orcinol **1**, as shown in Figure 12, it resulted in the synthesis of daurichromenic acid **5** in three steps (34). Compound **1** has previously been used as a starting material for the synthesis of **5** but it required five steps to accomplish the synthesis (32). First step was similar to the study of these researchers which they did in the year 2005. The **2** was refluxed with **1** in the presence of ethylenediamine diacetate (10

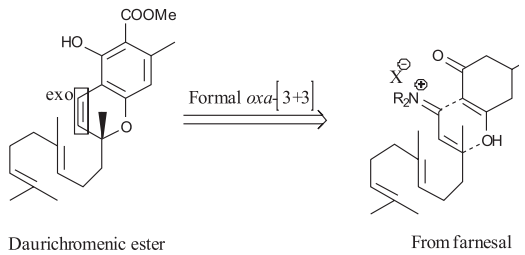


Figure 13. Synthesis of daurichromenic acid by formal *oxa*-[3+3] cycloaddition

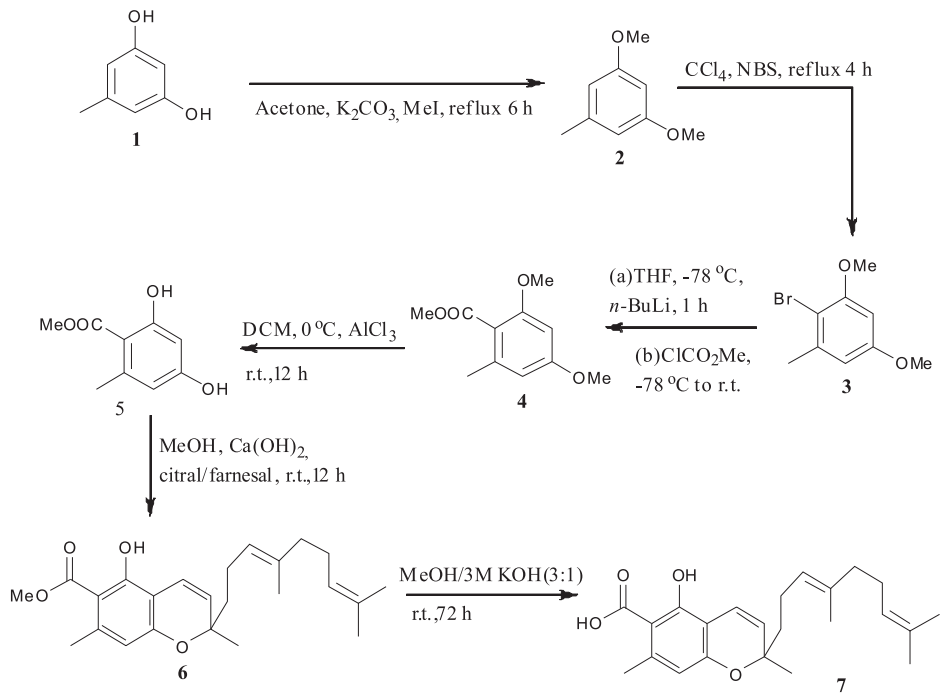


Figure 14. Synthesis of daurichromenic acid **7** from orcinol **1**. NBS = N-bromosuccinimide

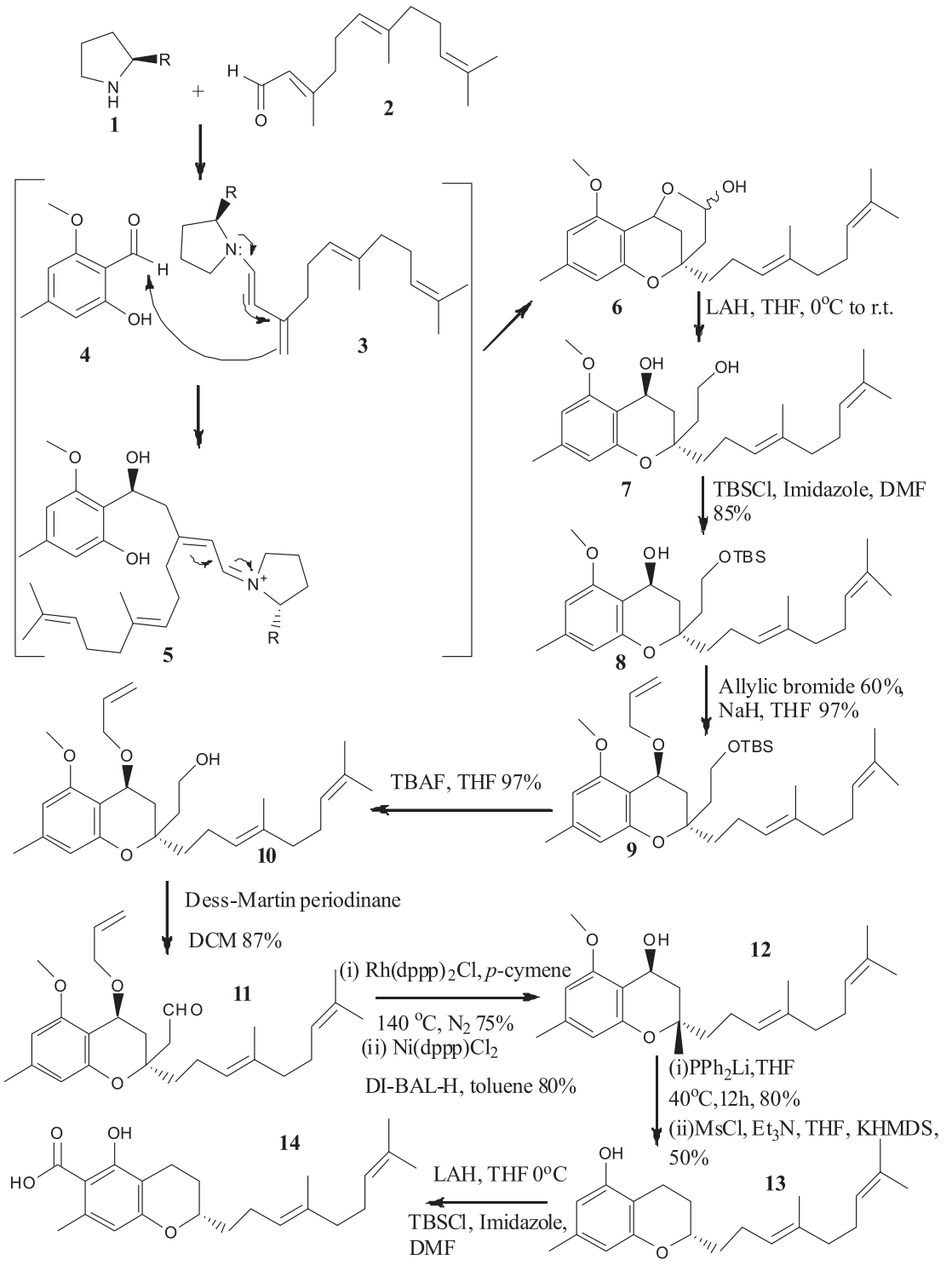


Figure 15. Enantioselective synthesis of daurichromenic acid **14**. Reagents used: LAH = lithium aluminum hydride, THF = tetrahydrofuran, TBSCl = tert-butyldimethylsilyl chloride, DMF = dimethylformamide, NaH = sodium hydride, TBAF = tetra-n-butylammonium fluoride, DCM = dichloromethane, $Rh(dppp)_2Cl$ = [1,3-bis(diphenylphosphino)propane]rhodium(II) chloride, $Ni(dppp)Cl_2$ = [1,3-bis(diphenylphosphino)propane]dichloronickel(II), DI-BAL-H = diisobutylaluminium hydride, $PPh_2Li$ = lithium diphenylphosphide, MsCl = mesyl chloride, $Et_3N$ = triethylamine, KHMDS = potassium bis(trimethylsilyl)amide

mol%) in xylene for 5 h. It yielded (±)-confluentin **3** (yield = 65%). In the next step, formylation of (±)-confluentin **3** was carried out in the presence of oxalyl chloride/DMF in acetonitrile at 0°C. The yield of the resultant compound **4** was 40%. The oxidation of **4** at room temperature for 10 h with buffered sodium chlorite gave **5** (yield = 78%). The spectroscopic data of **5** were in agreement with the literature (34).

Another approach adopted to synthesize the daurichromenic ester was formal *oxa*-[3+3] cycloaddition (36, 54). The retrosynthetic approach is shown in Figure 13. Grifolic acid is a natural product isolated from a fungus *Albatrellus dispansus*. Grifolic acid has been used to synthesize (±)-daurichromenic acid in just one step in the presence of dichlorodicyanobenzoquinone in benzene (yield = 73%). The reaction occurs at 90°C and with stirring time of 30 min. HPLC technique helps to separate (+)-daurichromenic acid and (-)-daurichromenic acid synthesized (38).

In another study, orcinol monohydrate **1** was used as a startng material to synthesize daurichromenic acid **7** (Fig. 14). This **1** was subjected first to methylation to produce **2** and then to N-bromosuccinimide-induced nuclear bromination to yield **3**. Next, lithiation followed by treatment with methyl chloroformate yielded an ester **4** which was demethylated, in the presence of $AlCl_3$, to produce the required phenolic ester **5**. Finally, benzopyran **6** was obtained from **5** by treating it with citral/farnesal in the presence of calcium hydroxide. A possible calcium ion complexation with the phenol groups can be the foundation of the observed regioselectivity. Saponification of **6** resulted in the production of **7** (reported yield 80%) (55). The reaction scheme is in Figure 14. The quest to synthesize daurichromenic acid led to another study in the year 2010. The speciality of this work was the enantioselective synthesis of daurichromenic acid (35). The scheme followed can be seen in Figure 15. In the first step, farnesal **2** and salisaldehyde **4** were reacted in the presence of a derivative of proline **1** to synthesize a lactol **6**. The **1** used was of configuration 'S' as only 'S' configured **1** can yield 'S' configured **6**. Once the 'S' configured **6** was obtained, it was reduced to a diol **7**. This **7** was protected selectively to yield **8** and was reacted with allyl bromide to synthesize **9**. Next, deprotection step (of OTBS) resulted in **10** and it was followed by oxidation to yield an aldehyde **11** which was decarboxylated and then deallylated to yield benzylic alcohol **12**. Demethylation of **12** resulted in an enantioselective chromen. Free phenol bromination at *ortho* position of this chromen followed by carboxylation yielded enantioselective daurichromenic acid **14** (35).

## CONCLUSION

Beyond various compounds, explored for anti-HIV activity, daurichromenic acid has been reported with excellent behavior. A high therapeutic index (TI) of daurichromenic acid proves its efficacy. Unlike other commercial anti-HIV products, it has been reported with no adverse side effects. Its pharmacological importance influenced the scientists to work on different synthetic approaches.

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# ANTIOSTEOPOROTIC EFFECT OF THE RHIZOME OF *DRYNARIA FORTUNEI* (KUNZE) (POLYPODIACEAE) WITH SPECIAL EMPHASIS ON ITS MODES OF ACTION

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**Abstract:** One of the elderly age health issues is a silent epidemic, the osteoporosis. Beside the extensive chemical investigations, the antiosteoporotic studies of various plants and their constituents have been carried out. The dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. (termed as Rhizoma *Drynariae*) is generally adopted in traditional therapy systems to treat osteoporosis. In addition to stimulatory role in bone formation, the current studies have proposed inhibitory effect of Rhizoma *Drynariae* on bone resorption. The pharmacological data of the extracts and total flavonoid contents of Rhizoma *Drynariae* have been discussed in this review article to summarize various modes of action of Rhizoma *Drynariae* and its compounds against osteoporotic models of osteoblasts, osteoclasts and animals. Being a player in bone resorption, cathepsin k is an important target molecule for managing osteoporosis through the use of Rhizoma *Drynariae* and its total flavonoids. Conclusively, further investigations should be conducted to develop future drug of choice using Rhizoma *Drynariae* for clinical treatment of osteoporosis.

**Keywords:** Rhizoma *Drynariae*, tissue engineered cartilage, cartilage regeneration, flavonoid, osteoporosis

One of the serious systemic skeletal diseases is osteoporosis, which emerges owing to many etiologies including hypogonadism, hypersteroidism, and menopause (1, 2). In osteoporosis, mineral density of bone tissue becomes significantly low, which results in deteriorated and fragile bones. Such bones are extremely prone to breakage (3).

Bone physiology depends on the delicate balance between bone resorption and regeneration. Bone resorption depends on deterioration of osteoblasts and osteoclasts through the process of apoptosis. Reversibly, bone regeneration involves the uninterrupted supply of new osteoblasts and osteoclasts (4). The functions of osteoblasts and osteoclasts are principally related with increased consumption of glucocorticoids, decreased level of sex steroids and senescence. Additionally, the prolonged lifespan of osteoclasts and shortened lifespan of osteoblasts leads to disturbance in equilibrium between bone resorption and regeneration.

An upregulation in osteoclastogenesis and osteoblastogenesis in the marrow has been observed in people with decreased level of sex steroids under the effect of upregulated levels and influence of cytokines, such as IL-1 and IL-6 (5). In addition, there is diminished bone formation in both aged male and female during each remodeling cycle. Furthermore, there is significant increase in the rate of bone remodeling in menopausal women (6).

On excessive usage of glucocorticoids, there is reduction in the intestinal absorption of calcium. Moreover, glucocorticoid excess also induces defects in the metabolism of vitamin D. Both of these changes lead to hypercalciuria and various pathological changes, including increased resorption

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of bones as well as decreased proliferation of osteoblasts. As a result, reduction in the biosynthesis of various macromolecules occurs resulting in deficiency of sex steroids, this situation escorts to hyperparathyroidism (7). Additionally, there is increased apoptosis as well as reduced synthesis of osteoblasts and osteocytes, on excessive usage of glucocorticoids (8).

The factors responsible for osteoporosis development include modifiable and non-modifiable factors. The former category involves medication use, calcium and vitamin D usage, sex hormones, anorexia nervosa, alcohol consumption, and smoking, while age, gender, body size, family history, and ethnicity are named as non-modifiable factors (2). The osteoporosis can be prevented or treated through daily exercise, balance diet, and various drugs. Estrogen, bisphosphonates, calcitonin, and sodium fuoride are the drugs which are used to treat osteoporosis (9).

The usual therapeutic approaches for managing osteoporosis focus on the remodeling of bones through various modes involving the provision of estrogens, calcium and phosphorus in bones; the stimulation of parathyroid hormone (PTH) synthesis; the induction of OPG (osteoprotegerin) expression, osteoblast proliferation, and the osteoclast apoptosis; and reduction in the level of IL-1, 4, and 6 (10).

Therapies that play important role in reducing bone resorption include vitamin D, calcium, hormone, and bisphosphonate. Little therapeutic outcome has been noted in terms of improved bone mineral density when vitamin D or calcium alone is supplemented in deficient persons (10). Hormone therapy is another approach for managing the osteoporosis in the postmenopausal women, however it should be dealt as a short-term therapy since its long-term use may lead to development of breast and/or uterus cancer (11). Along with the preventive effect against osteoporosis, bisphosphonate intake produces some undesired effects in upper gastrointestine that may persist for many years after their discontinuation (12). For example, potential undesired effect of estrogen is an increased risk of venous thrombosis (13). Additionally, therapies that play important role in stimulating the bone regeneration include PTH, which is involved in bone anabolism. At a dose of 20 μg, PTH (1-34) effectively inhibited fracture risk in postmenopausal women. However, the discontinuity of PTH is followed by decline in bone mineral density (14). This drawback of PTH has been managed by using an antiosteoporotic compound, strontium ranelate after discontinuing PTH (15, 16).

The above given summary of synthesized drugs used for preventing and treating the osteoporosis elaborates their specific drawbacks. In comparison to these drugs, some natural therapeutic agents from various medicinal plants are found to possess promising antiosteoporotic activity with lesser side effects, even after long-term use. It is noteworthy that the plant medicines possess plenti-

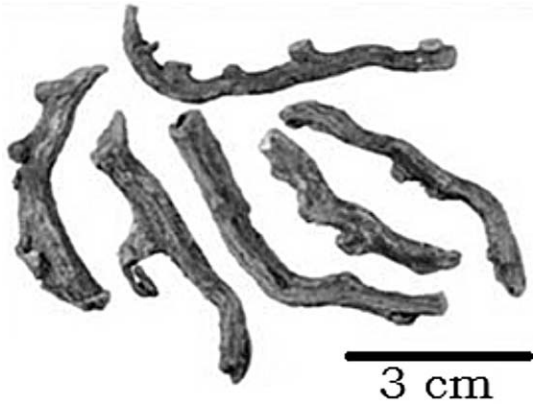
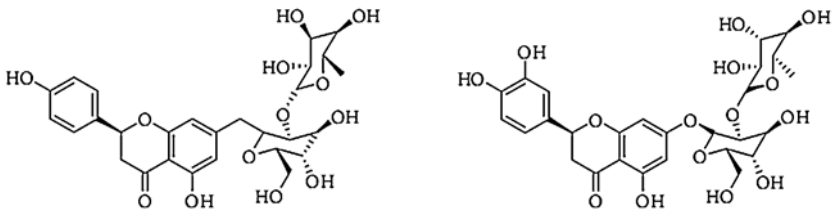



Figure 1. Rhizoma *Drynariae*



Naringin Neoeriocitrin

Figure 2. Chemical structure of naringin and neoeriocitrin, isolated from *Drynaria fortunei* (20)

ful active compounds. It is useful in treating osteoporosis, which is a disease with multipathways and multitargets of pathogenesis (16).

*Drynaria fortunei* is a well known medicinal plant whose dried rhizome is extensively used for treatment of bone diseases, inflammation, and hyperlipemia (17). It is especially used in Traditional Chinese Medicines (TCM). *Drynaria fortunei* (Kunze) J. Sm. is a perennial pteridophyte that belongs to family, Polypodiaceae (18). Due to use of dried rhizome of *Drynaria fortunei* in disease therapy, the term "Rhizoma *Drynariae*" is generally used for this herbal drug. In TCM, Rhizoma *Drynariae* is also called as "Yang-tonifying" herb; it means herb used to treat bone diseases (18, 19). Moreover, various flavonoids and triterpenoids are found in Rhizoma *Drynariae* extract, and these compounds are considered to be responsible for improving the bone cell viability (20). Naringin and neoeriocitrin are leading examples of flavonoids isolated from Rhizoma *Drynariae* extract (18). These compounds have antiosteoporotic activity, possibly due to its capability of activating the estrogen receptors as well as replacing estrogen (21). The important examples of triterpenoids isolated from Rhizoma *Drynariae* extract include 24-ethyl-9,19-cyclolanost-25-en-3; 3-ol, hop-22(29)-ene and fern-9(11)-ene, respectively (18). Naringin has been demonstrated to possess the capability of stimulating new bone formation (20). Figure 1 reveals the structure of Rhizoma *Drynariae*. In addition, Figure 2 describes the chemical structure of naringin and neoeriocitrin, isolated from *Drynaria fortunei* (20).

The pharmacological data of the extracts, serum, and Rhizoma *Drynariae* total flavonoids has been found from literature search about various modes of action of Rhizoma *Drynariae* and its compounds against osteoporotic models of osteoblasts, osteoclasts and animals (22-27). For example, significant increase in alkaline phosphatase activity in the cell lines, enhancement in proteoglycan synthesis, and improvement in calcification of the cultivated chick embryo bone primordium is observed after Rhizoma *Drynariae* injection (28). After treatment of rat osteoblasts with Rhizoma *Drynariae*, considerable bone recovery *via* its antioxidant action has been reported (29). A biochemical study has reported significant effect of Rhizoma *Drynariae* on osteclastic cell lines (23, 30).

This review article reports the summary of current studies about antiosteoporotic potential of the dried rhizome of *Drynaria fortunei* with special focus on its mode of action.

## ANTIOSTEOPOROTIC POTENTIAL OF THE DRIED RHIZOME OF *DRYNARIA FORTUNEI*

The Rhizoma *Drynariae* has effectively been used for many years in the eastern Asia (especially in China and Korea) as an anti-inflammatory, hypolipemic, anti-arteriosclerotic, and anti-osteoporotic agent (31-33). Numerous current studies elaborate therapeutic effectiveness of *Drynariae* Rhizoma in osteoporosis and bone fracture in various models such as the ovariectomized rat model. The modes of action of Rhizoma *Drynariae* include stimulation of BMP-2 and ALP (alkaline phosphatase), aggregation of bone matrix proteins such as type I collagen, and increased expression of up-regulated Runx2 and osteocalcin (34). Beside Rhizoma *Drynariae* total flavonoids, Rhizoma *Drynariae* has been used for bone treatment in different forms including Rhizoma *Drynariae* medicated-extracts and serum.

### Extracts

The mouse bone cells culture was exposed to various dilutions of *Drynariae* Rhizoma extract to investigate its anti-resorption potential. From colorimetric MTT assay conducted for determination of mitochondrial activity of these cells, significant anti-resorptive effect of *Drynariae* Rhizoma extract was observed. It is noteworthy that no cytotoxicity was observed in this experiment. This outcome is in agreement with another report (35). A dilution of 100 µg/mL of *Drynariae* Rhizoma extract produced maximum bone protective effect. Alternatively, osteoclasts contain cathepsin k, an endoproteinase that is found largely in the lysosomes, which is considered to be responsible for bone resorption *via* matrix degradation. Cathepsin k is present only in the osteoclasts. Cathepsin k expression can be inhibited by gene silencing, leading to inhibition of bone resorption as well as the collagen decomposition (36), while cancellous bone turnover is observed when cathepsin k overexpression. Thus, cathepsin k may be considered as a specific biomarker in bone resorption. These information suggest that cathepsin k may be a crucial target (37, 38).

Another experiment involving the treatment of mouse long bone cells such as osteoclasts and osteoblast with wortmannin (the PI3-kinase inhibitor) and calphostin C (a specific inhibitor of protein kinase C), was conducted. The results revealed the inhibitory effect on the osteoclast-reconciled intracellular dispensation of cathepsin k. Similar findings were obtained when *Drynariae* Rhizoma extract was used instead of wortmannin. On the other hand, mannose-6-phosphate receptor

is involved in the re-entrance of the secreted proenzymes into cells. Thus, one more experiment was carried out to study the inhibitory effect of wortmannin and *Drynariae* Rhizoma extract on the possibility of this re-entrance in the absence or presence of mannose-6-phosphate. The results revealed the dose-dependent inhibitory effect on the osteoclast-reconciled intracellular dispensation of cathepsin k. Additionally, an elevated level of wortmannin and *Drynariae* Rhizoma potency was observed in the presence of mannose-6-phosphate. Conclusively, dose-dependent inhibition of *in vitro* bone resorption and cathepsin k processing by *Drynariae* Rhizoma was noted. In this way, *Drynariae* Rhizoma extract can be taken as a prodrug with bone resorption inhibiting feature, since it possesses capability of ceasing the maturing process of cathepsin k in osteoclasts-containing long bone cells (39). Later on, Jeong et al. (34) studied the effect of *Drynariae* Rhizoma extract on bone tissue formation, ossification, using MC3T3-E1 (non-transformed osteoblasts) and rat bone marrow cells. As a result, *Drynariae* Rhizoma was found to be involved in stimulation of the ALP activity and mineralization in a dose-dependent in a concentration range of 50-150 µg/mL. Moreover, significantly elevated levels of bone morphogenetic protein-2 and ALP mRNA were noted at a concentration of 100 µg/mL of *Drynariae* Rhizoma. Additionally, non-significant elevated levels of type I collagen mRNA were noted at a concentration of 60 µg/mL of *Drynariae* Rhizoma, leading to the gene expression inhibition of collagenase-1 during 15-20 days of culture. Decisively, these anabolic effects of *Drynariae* Rhizoma results in an increase in proliferation and differentiation of osteoblasts *in vitro*, revealing the bone protective activity of *Drynariae* Rhizoma (40).

In order to verify the *in vitro* results that Rhizoma *Drynariae* extract promotes *in vitro* bone cell viability through an increase in intracellular total protein, alkaline phosphatase and acid phosphatase (23), the systemic effect of Rhizoma *Drynariae* extract on bone structure in normal mice has been examined using micro-CT scanning. Wong and Rabie used 8 week old male BALB/c mice to compare the bone structures of mice treated with and without Rhizoma *Drynariae* extract. The results of this *in vivo* study show that there is 0.25 mm enhancement in the proximal end of the left tibia of each mouse. The quantitative morphometric analysis of the bone structures revealed that there was an increase in the bone density as evident from augmented bone volume/tissue volume ratio and bone trabeculae by 6.45% and 10.00%, respectively, in Rhizoma *Drynariae* extract treated mice. It could be concluded from these results that the bone density can be improved by oral intake of Rhizoma *Drynariae* extract. This *in vivo* study supported the previous *in vitro* findings about the anabolic effect of Rhizoma *Drynariae* extract, i.e., this extract enhanced the bone cell activity (23, 41). In another study to test bone strengthening activity of Rhizoma *Drynariae*, Wong et al. investigated the systemic effect of Rhizoma *Drynariae* extract on bone formation in eight week old male BALB/c mice. The results showed that there is enhancement in the trabecular number and bone density by 10% and 6.45%, respectively, which reveals the change in bone histomorphology in Rhizoma *Drynariae* extract treated mice as compared to control group of these animals. As evident from the identification of osteoblasts and osteocytes in the newly formed bone, it was also noted that there was induction of new bone formation on the margins of the defects in Rhizoma *Drynariae* treated mice. It indicates the systemic effect of this extract on bone formation and healing. In addition, this activity of Rhizoma *Drynariae* could be due to its active constituent, naringin. Naringin has been demonstrated to be involved in the up-regulation of osteogenic factor expression, which results in angiogenesis and/or osteogenesis. As concluding remarks, the osteogenic effect of naringin and other active ingredients of Rhizoma *Drynariae* on fracture-prone bones including femur neck and lumbar spine may also be investigated (42).

**Serum**

The influence of serum medicated with different doses of RDTF (Rhizoma *Drynaria* total flavonoids) on the osteoblasts of newborn SD rats cultured by collagenase method, was investigated. These cells were then tested for MTT, PNPP, PI and Annexin V/PI analysis and it was observed the significantly increased proliferation and alkaline phosphatase activity in RDTF treated osteoblasts than that of the untreated osteoblasts, demonstrating the anti-osteoporosis activity in a time dependent manner (43).

**Rhizoma Drynaria total flavonoid extract**

RDTF are obtained through extraction of dry rhizomes of *Drynaria fortunei*, followed by isolation and purification (44). Naringin is the main active constituent of RDTF. The RDTF play an important role in treatment of the bones with lesions, low density and strength, increased blood viscosity, low

bone mineralization, and resorption especially postmenopausal osteoporosis (39, 45-49).

Kang et al. prepared water and ethanol extract of *Drynariae* Rhizoma to compare the extraction efficacy of total flavonoids. Moreover, both extracts were tested *in vitro* for comparing their antioxidant and anti-osteoporosis features. The ethanol extract showed better extraction efficacy and antioxidant activity in comparison to that of water extract. Additionally, the ethanol extract showed better proliferation and differentiation of cultured mouse (KP100 CD-1) osteoblastic cells *in vitro*. Based on these results, it can be concluded that the ethanol extract of *Drynariae* Rhizoma might be more effective for osteoporosis treatment (50).

In postmenopausal osteoporosis, Rhizoma *Drynariae* is capable of sustaining the normal trabecular structure as well as inhibiting rate of bone turnover through mimicking the estrogen. In addition, RDTF possess potent anti-oxidative and osteoprotective activity, possibly through its potential of restoring OVX-induced osteoporosis in rats. Moreover, the increased osteoblasts proliferation as well as the reduced osteoclasts activity in rat bones-treated with RDTF, was observed *in vitro* (51).

Smad is a protein that plays a role in intracellular signaling for silk threonine kinase receptors type I and II, which are involved in the generation of bones. In order to examine the influence of RDTF on expression of the Smad1 and Smad5 mRNA, Zhu et al. (52) administered RDTF to the ovariectomized rats (except normal group consisting of non-ovariectomized rats) in high, moderate, and low doses. The femur bone assays of the sacrificed rats showed that Smad1 and Smad5 were more expressed in RDTF treated rats, as compared to normal group rats. This effect was dose independent (52).

Pang et al. (53) conducted a study to explore the bone protection in young ovariectomized (C57/BL6J) mice by using RDTF based on its estrogen-like activity. The maximum increase in trabecular-rich bone mineral densities at distal femur and lumbar spine in the ovariectomized mice treated with 0.173 mg of RDTF per gram mice weight per day, was observed. The peripheral quantitative computed tomography approach was employed to study bone mineral densities. The co-incubation of RDTF with 17β-estradiol antagonist ICI 182, 780 in rat osteoblast-like UMR-106 cells did not show stimulation of osteoblasts by RDTF; it showed ER-dependent osteoblastic functions of RDTF. Moreover, transient transfection in UMR-106 cells was also studied. It revealed that ERE-dependent luciferase activity depended on dose of RDTF through ER-α and ER-β. Conclusively, RDTF not only activates ER leading to growth regulation of the osteoblasts, but also protects mice from ovariectomy-induced osteoporosis. Additionally, the postmenopausal women with osteoporosis should also be clinically treated to assess therapeutic efficacy of RDTF. Such results were already published by Liu et al., demonstrating that RDTF-3H-TdR mixture promotes the alkaline phosphatase activity in UMR-106 cells in a dose- and time-dependent manner in osteoblast culture *in vitro* (54).

In order to provide experimental confirmation about the mode of Rhizoma *Drynariae* for osteoporosis treatment, an interventional study was conducted to study the effect of RDTF on the osteoclasts through detection of bone mineral density, biomechanics, serum cathepsin k concentration, and cathepsin k mRNA expression in the proximal metaphysis of the tibia in 72 female Sprague-Dawley ovariectomized rat models of osteoporosis. Additionally, a three-point bending approach was used for measuring the maximum load of the tibia to assess the influence of RDTF on bone strength. In comparison to that in the estrogen and normal groups, there were significant differences in the bending load, serum cathepsin k concentrations, and cathepsin k mRNA expression in the RDTF rats. Moreover, RDTF showed a concentration-dependents effect on bone mineral density (55). In a study about interventional effect of RDTF on cathepsin k, bone density of the ovariectomized rat model with osteoporosis under treatment using RDTF was determined and found RDTF mediated-increase in bone density, in comparison to the control group (56-60).

**Isolated compound - naringin**

The leading example of flavonoid isolated from Rhizoma *Drynariae* extract is naringin that plays an important role in bone metabolism and osteogenesis (39, 61, 62). It is capable of suppressing the retinoic acid-provoked osteoporosis in rats; enhancing expression of BMP-2 resulting in bone formation; increasing the proliferation and osteogenic differentiation of bone mesenchymal stem cells in osteoporosis diseases in human (63-65). Due to double directional adjusting effect (i.e., estrogenic and anti-estrogenic functions), naringin is found to manage osteoporosis *via* selectively binding with estrogenic receptor (66, 67). In addition, current studies have proved the usefulness of naringin in ovariectomized-induced bone loss in mice/rats (68-71).

Table 1. Clinical trials using Rhizoma *Drynariae* for treating osteoporosis.

| No. | Treatment | No. of subjects | Treatment group | Treatment duration (days) | Bone mass density change ($g/cm^2$) | Adverse effects (%) | Ref. |
|---|---|---|---|---|---|---|---|
| 1 | *Rhizoma Drynariae* | 34 | PT | 180 | +0.005 (lumbar); +0.025 (femur) | Not observed | 73 |
| | Tibolone | 35 | HT | | +0.043 (lumbar); +0.051 (femur) | Uterine bleeding (8.6%) | |
| 2 | *Rhizoma Drynariae* | 48 | PT | 180 | +0.024 (femur) | Not observed | 74 |
| | Estradiol valerate, medroxyprogesterone | 42 | HT | | +0.015 (femur) | | |
| 3 | *Rhizoma Drynariae* | 40 | PT | 180 | +0.038 (femur) | Not observed | 75 |
| | Nilestriol | 40 | HT | | +0.039 (femur) | | |
| 4 | *Rhizoma Drynariae* | 50 | PT | 90 | +0.103 (forearm) | Not observed | 76 |
| | Nilestriol | 30 | HT | | +0.056 (forearm) | | |

**Clinical trials using Rhizoma *Drynariae* for treating osteoporosis**

The fundamental remedies for osteoporosis include bisphosphonates and estrogen, but these agents have significant side effects. Therefore, natural remedies are currently being investigated, particularly from plants, since possibility of finding of natural ingredients is significantly higher in plants than indiscriminate prospect in conventional approaches, as evident from their folk use in osteoporosis. In contrast to the admired view "herbals are natural and harmless", herbal safety is of prime importance, since some herbals have been reported to have serious side effects including hepatotoxicity. Thus, clinical usage of herbal drugs should be recommended after thorough quality control testing and standardization. Table 1 reflects the clinical trials using Rhizoma *Drynariae* for treating osteoporosis. It is clearly evident from most of the studies that the anti-osteoporotic activity of Rhizoma *Drynariae* is comparable to various standards including tibolone, estradiol valerate, medroxyprogesterone, and nilestriol, in terms of bone mass density change during study for 180 days. Moreover, these studies reported no adverse effects of Rhizoma *Drynariae*, except for tibolone that resulted in uterine bleeding (8.6%) (72).

## CONCLUSION

The literature study proposes various modes of anti-osteoporotic activity of RDTF as given here: Firstly, RDTF promoted the proliferation of MCF-7 (human breast cancer cell line) and ROS17/2.8 (osteoblast-like cell line), even more efficiently than estradiol and genistein showing the estrogen-like effect of RDTF. Secondly, RDTF inhibited the lacunae production in osteoclasts leading to inhibition of cellular expression of cathepsin k. Finally, RDTF inhibited transport and expression of the cathepsin k precursor in cells, with the same effectiveness as that of wortmannin. These studies verify that RDTF treats osteoporosis through inhibition of cathepsin k.

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## ANALYSIS

# APPLICATION OF GLASSY CARBON ELECTRODE MODIFIED WITH NAFION/MWCNTS FOR SENSITIVE VOLTAMMETRIC DETERMINATION OF THYMOL

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**Abstract:** The glassy carbon electrode modified with Nafion and multi-walled carbon nanotubes (Nafion/MWCNTs), applied for the determination of thymol using differential pulse voltammetry in phosphoric acid and methanol is presented. The calibration graph obtained for thymol is linear from 0.5 µM (75 µg/L) to 5 µM (750 µg/L) for a preconcentration time of 15 s, with correlation coefficient of 0.998. For a GC-Nafion/MWCNTs electrode the detection limit for a preconcentration time of 30 s is as low as 7.5 µg/L. The repeatability of the method at a concentration level of the analyte as low as 75 µg/L, expressed as RSD is 3.9% (n = 5). The proposed method was successfully applied and validated by studying the recovery of thymol from urine, dental mouthwash and liquid anti-ace.

**Keywords:** thymol, MWCNTs, Nafion, voltammetry

Thymol (2-isopropyl-5-methylphenol) is a natural monoterpene phenol derivative of cymene, $C_{10}H_{14}O$, isomeric with carvacrol. It has been reported many plants which contain thymol as major component like: *Thymus vulgaris*, *Origanum vulgare*, *Trachyspermum ammi* and many others (1-5). Thymol is an active ingredient in pesticide products registered for use as animal repellents, fungicides/fungistats, medical disinfectants, tuberculocides, and virucides. These products are used on a variety of indoor and outdoor sites, to control target pests including animal pathogenic bacteria and fungi, several viruses including HIV-I, and birds, squirrels, beavers, rats, mice, dogs, cats and deer. Thymol also has many non-pesticidal uses, including use in perfumes, food flavorings, mouthwashes, pharmaceutical preparations and cosmetics (6). Thus a sensitive, specific, fast and cheap method of determining thymol is necessary for studying presence of thymol in various medical samples.

A number of analytical methods have been reported for the determination of thymol such as: gas chromatography (7-9), high performance liquid chromatography (10, 11), thin-layer chromatography (12), multivariate response surface methodology (13) and flow injection spectrophotometry (14). However, these methods are usually time consuming and require complicated pretreatment. On the other hand, voltammetric techniques are rapid, relatively chip and highly sensitive. In the group of voltammetrics methods various working electrodes for the determination of thymol such as: glassy carbon electrode (15), single-walled carbon nanotubes screen-printed electrodes (16), $CeO_2$ nanoparticle-decorated graphene hybrid film electrode (17) are used.

The aim of this work was to study the high sensitive determination of thymol by means of linear sweep voltammetry (LSV) and differential pulse voltammetry (DPV) with the use of glassy carbon (GC) electrode modified with Nafion/multi-walled carbon nanotubes (Nafion/MWCNTs). The new procedure was examined and successfully utilized for the determination of a low thymol concentration in urine, dental mouthwash and liquid anti-ace. Potential interferences from selected metal ions, citric acid and surface-active substances were checked.

## EXPERIMENTAL

### *Apparatus and software*

A multipurpose Electrochemical Analyzer M161 with the electrode stand M164 (both MTM-

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ANKO, Poland) were used for all voltammetric measurements. The classical three-electrode quartz cell, volume 20 mL, consisting of a GC electrode (diameter 3 mm, Mineral, Poland) modified with Nafion/MWCNTs as the working electrode, a double junction reference electrode Ag/AgCl/KCl (3 M) with replaceable outer junction (3 M KCl) and a platinum wire as an auxiliary electrode were used. pH measurements were performed with laboratory pH-meter (N-512 elpo, Polymetron, Poland). Stirring was performed using a magnetic bar. All experiments were carried out at room temperature. The MTM-ANKO *EAGRAPH* software enabled electrochemical measurements, data acquisition and advanced processing of the results.

**Chemicals and glassware**

All reagents used were of analytical grade. $KH_2PO_4$, $K_2HPO_4$ were obtained from Merck and $H_3PO_4$ was obtained from CHEMAN (Poland). In measurements a 0.1 M phosphate solutions were used. Standard stock solutions of thymol (0.01 M) were prepared by dissolving thymol (local source) in methanol. Solutions with lower thymol concentrations were made by appropriate dilution of the stock solution. The multi-walled carbon nanotubes (purity >95%, diameter 40-60 nm, length 5-15 μm) were obtained from Nanostructured & Amorphous Materials Inc. (USA). Nafion 5 wt. % solution in a mixture of lower aliphatic alcohols and water was purchased from Aldrich.

Prior to use, glassware were cleaned by immersion in a 1 : 1 aqueous solution of $HNO_3$, followed by copious rinsing in distilled water.

**Preparation of the electrode**

Prior to modification, the GC electrode was mechanically polished with $Al_2O_3$ (0.05 μm), and then rinsed and sonicated 5 min in distilled water. Next, 10 mg of MWNTs was added to 10 mL ethanol and Nafion (final Nafion concentration 0.1%), and then sonicated for 2 h to obtain homogenous suspension. The prepared GC electrode was coated with 10 μL of homogenous Nafion/MWCNTs and allowed to evaporate the solvent at room temperature in the air.

**Standard procedure of measurements**

The electrochemical behavior of the Nafion/MWCNTs glassy carbon modified electrode was investigated using cyclic voltammetry. The voltammograms were recorded in the potential range from -200 to 1350 mV. Before each registration scan, the potential of 1350 mV (3 s) was applied to clean the surface of the electrode. The electrode conditioned in this way was used to determine thymol in the supporting electrolyte: 0.1 M phosphoric acid (total volume 10 mL) contained in a quartz voltammetric cell. In the case of DPV measurements the potential of the electrode was changed in the following sequence: cleaning potential 1350 mV for 3 s and preconcentration potential $E_{acc}$ = -50 mV for $t_{acc}$ = 10 s. During the preconcentration step thymol was collected while the solution was being stirred (ca. 500 rpm) using a magnetic stirring bar. Then, after a rest period of 3 s, a differential pulse voltammogram was recorded in the anodic direction from 450 to 1350 mV. The other experimental parameters were as follows: step potential, 6 mV; pulse potential, 50 mV; time step potential, 40 ms (20 ms waiting + 20 ms sampling time). The measurements were carried out from undeaerated solutions. Quantitative measurements were performed using the standard addition procedure.

**Sample preparation**

**Urine**

For DPV determination of thymol in urine, 250 μL of the fresh sample was added directly into voltammetric cell with supporting electrolyte (total volume 10 mL).

**Dental mouthwash and liquid anti-ace**

For the determination of thymol in dental mouthwash and liquid anti-ace, at first the products were 10-fold diluted with methanol and next, 50 μL of the samples were added to the voltammetric cell.

## RESULTS AND DISCUSSION

**Cyclic voltammetry studies**

***Effect of the scan rate***

The influence of the scan rate ($v$) on the thymol peak at the GC electrode modified with Nafion/MWCNTs was investigated in the range of 10 mV/s to 500 mV/s (Fig. 1). The peak current *vs.* square root of scan rate (Fig. 2) gave a straight line up to 500 mV/s. The obtained linear regression equation is:

$$I_p = 2.7v^{1/2} - 1.1 \text{ [μA]}, \qquad r = 0.997$$

This suggests that the process of electrode reaction is controlled by diffusion of thymol.

The anodic peak potential was shifted in the positive direction with the increasing scan rate. The peak potential *vs.* ln scan rate gave a straight line (Fig. 3). The obtained linear regression equation is:

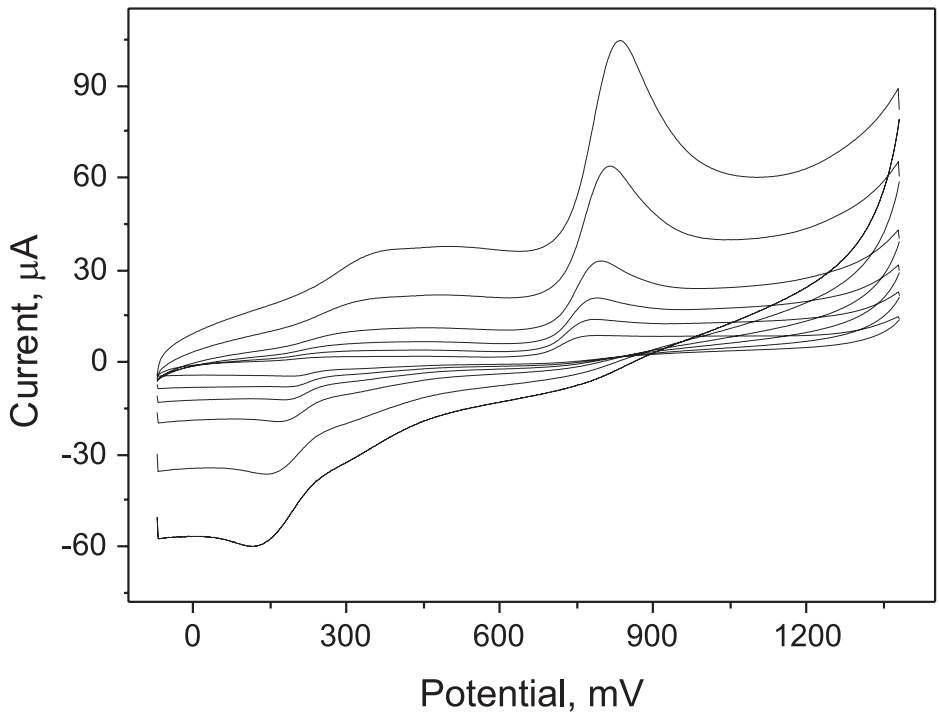


Figure 1. The cyclic voltammograms obtained for 0.25 mM thymol at the GC electrode modified with 10 μL Nafion/MWCNTs in 0.1 M $H_3PO_4$. Scan rate in the range from 10 to 500 mV/s

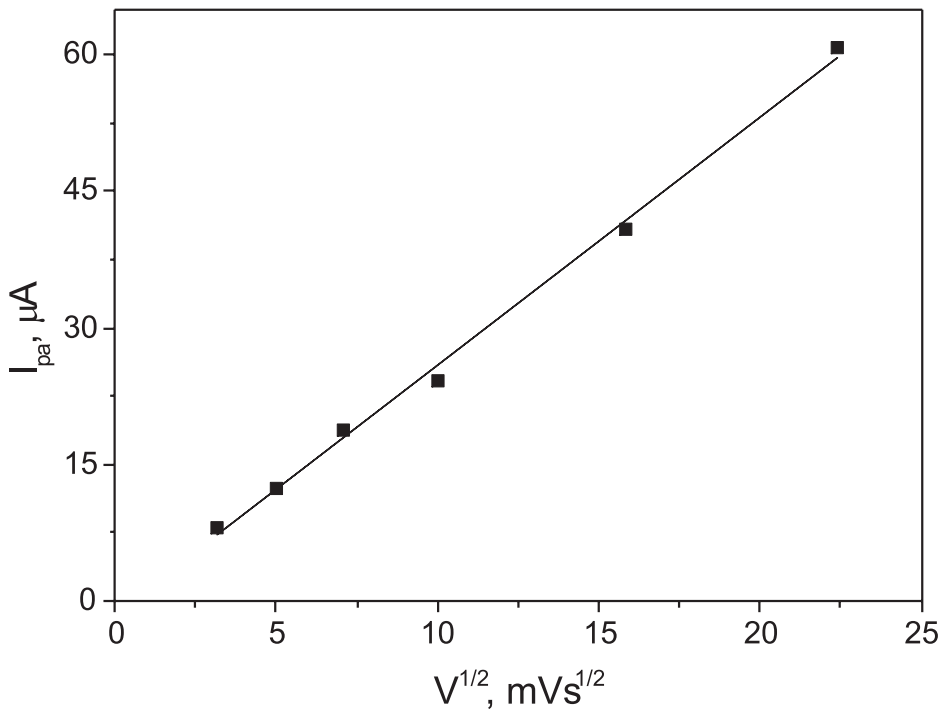


Figure 2. Dependence of the thymol peak current on square root of scan rate in the range from 10 to 500 mV/s for 0.25 mM thymol in 0.1 M $H_3PO_4$

$E_p = 20.3\ ln(v) + 747$ [mV], r = 0.997

Based on the theory for an irreversible electrode reaction the following equation holds (18):

$$E_p = E^0 + \frac{RT}{\alpha n F}\left[0.780 + \ln\left(\frac{D_0^{1/2}}{k_s}\right) + \ln\left(\frac{\alpha n F v}{RT}\right)^{1/2}\right] \quad (1)$$

where $E^0$ is the formal potential, a is the transfer coefficient, n is the number of electrons involved in the charge-transfer step, $k_s$ is the standard rate constant, F is the Faraday constant, D is the diffusion coefficient, R and T have their usual meaning. From the slope of $E_p$ *vs. ln(v),* $\alpha n = 0.63$ could be obtain

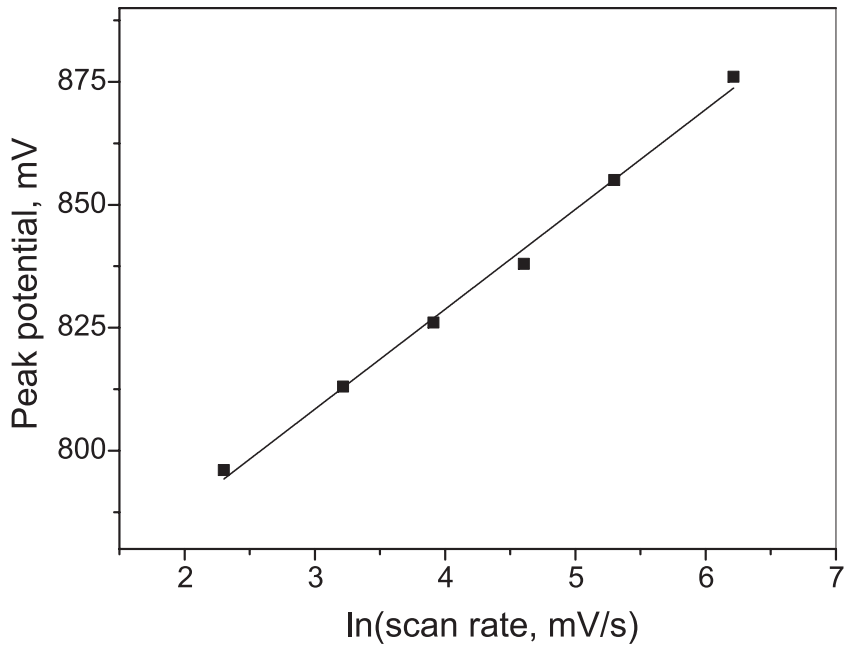


Figure 3. Dependence of the thymol peak potential on ln of scan rate in the range from 10 to 500 mV/s for 0.25 mM thymol in 0.1 M $H_3PO_4$

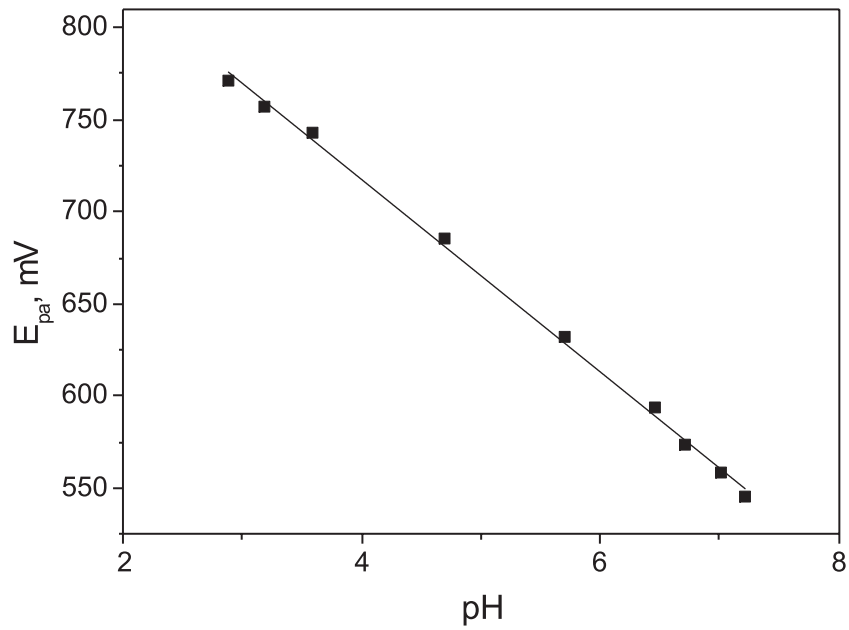


Figure 4. Dependence of the thymol peak potential on pH in the pH range from 2.9 to 7.2 for 0.25 mM thymol in 0.1 M phosphate buffers

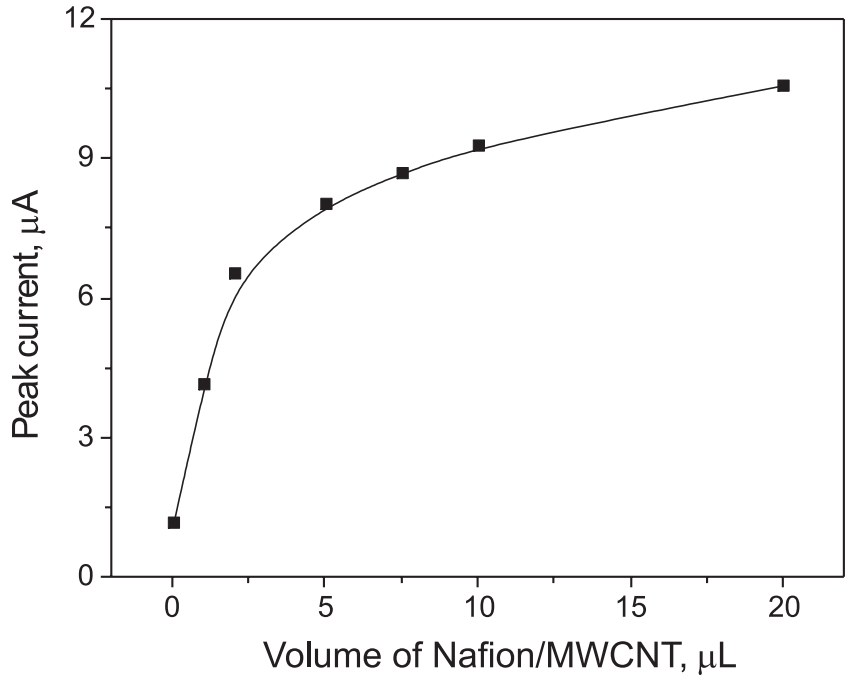


Figure 5. Dependence of the peak current on volume of Nafion/MWCNTs on GC electrode in the range of 0 to 20 μL for 25 μM thymol in 0.1 M $H_3PO_4$. Instrumental parameters: $\Delta E$ = 50 mV, $E_s$ = 6 mV, $t_w$, $t_s$ = 20 ms. Preconcentration potential -50 mV, preconcentration time 10 s, stirring rate 500 rpm

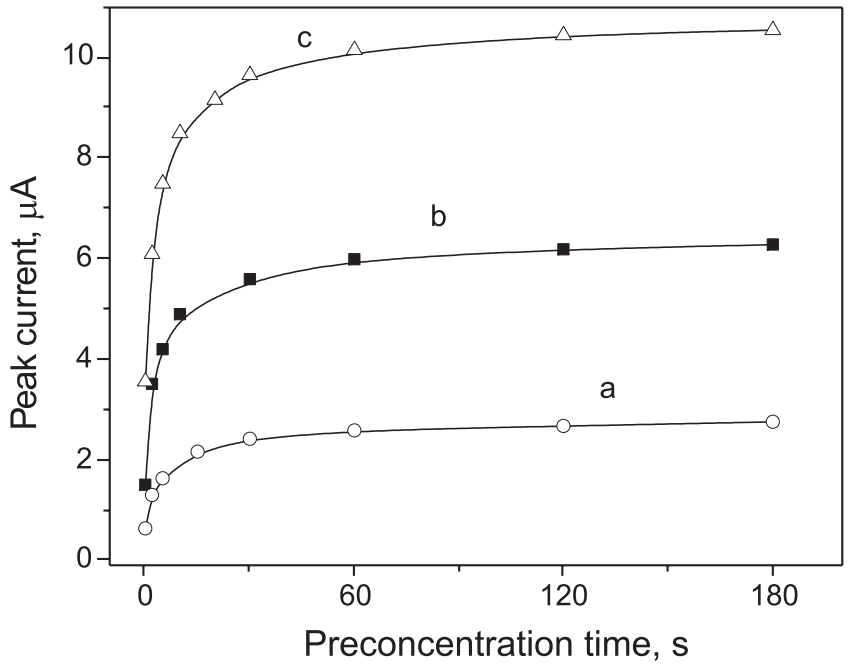


Figure 6. Dependence of the peak current on preconcentration time in the range from 0 to 180 s for (a) 2.5; (b) 10 and (c) 25 mM thymol in 0.1 M $H_3PO_4$. All other conditions are as in Figure 5

and the number of the electron transfer for α assuming 0.5 could be calculated to 1.

***Effect of the pH values***

The influence of the pH value on the peak potential at the GC electrode modified with Nafion/MWCNTs was investigated in the pH range from 2.9 to 7.2 (Fig. 4). The thymol peak potential shifted negatively as the pH increased, indicating that the electrode process involved proton participation. The dependence between the peak potential and the pH is linear and the obtained equation is:

$$Ep = -52.2\ pH + 926\ [mV], \quad r = 0.998$$

The slope of this relationship is close to the expected theoretic value of 59.1 mV/pH and suggests that the equal numbers of protons and electrons are involved in the electrode reaction.

**Influence of DPV parameters on technique on thymol peak**

The important parameters of the DPV technique are pulse amplitude ($\Delta E$), potential step amplitude ($E_s$), waiting time ($t_w$) and sampling time ($t_s$). Consequently, these parameters were investigated. To optimize the conditions for thymol measurements, the following instrumental parameters were systematically varied: *DE* in the range 5 - 100 mV (both positive and negative mode), $E_s$ in the range 1 - 7 mV, $t_w$ and $t_p$ from 10 to 60 ms.

The best results were obtained for the amplitude of 50 mV (the peak current was ~8.5 mA for 25 mM thymol). Higher pulse amplitude (>50 mV) caused major growth of the background current. For further work, the pulse amplitude of 50 mV was applied.

Changes of the step potential cause influence on peak current. For a step potential equal to 1 mV the peak current was 2.2 µA, and for a step potential of 7 mV the peak current was 9.4 µA. The step potential of 6 mV was applied in further work.

The waiting time and sampling time were changed in the range from 10 to 60 ms. The best result was obtained for waiting time and sampling time of 20 ms, and this was the value chosen for further work.

**Influence of the volume of Nafion/MWCNTs on thymol peak**

The mixture of Nafion/MWCNTs coated on the GC electrode is necessary to obtain high sensitive determination of thymol. The thymol peak current depends on the volume of Nafion/ MWCNTs (Fig. 5). For bare GC electrode the thymol peak current was 1.1 µA. The presence and increase of the amount of Nafion/MWCNTs on the GC electrode is accompanied by an increase of the thymol peak. The optimal volume of Nafion/ MWCNTs was for 10 µL (with the peak current reaching values about 9 mA

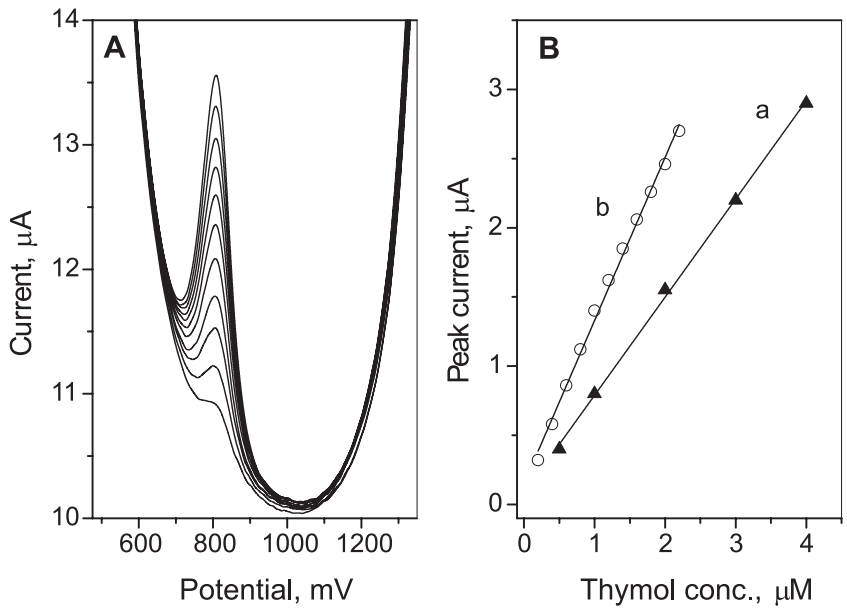


Figure 7. **A** – The DP SV thymol calibration voltammograms from 0.2 to 2.2 mM obtained for preconcentration time 30 s in 0.1 M $H_3PO_4$, **B** – thymol calibration curves obtained for preconcentration time: (a) – 15 and (b) – 30 s. All other conditions are as in Figure 5

Table 1. Results of thymol determination in various samples (n = 3).

| Thymol added | Thymol found $\bar{x}$ ± s (recovery %) | | | |
|---|---|---|---|---|
| | Urine (μM) | I - Dental mouthwash[1] (mg/100 mL) | II - Dental mouthwash[1] (mg/100 mL) | Liquid anti-ace[2] (mg/100 mL) |
| 0 | 0 | 62.3 ± 4.1 | 64.1 ± 2.9 | 51.1 ± 3.7 |
| 0.5 μM | 0.44 ± 0.05 (88) | - | - | - |
| 1.5 μM | 1.38 ± 0.11 (92) | - | - | - |
| 50 mg/100 mL | - | 116.7 ± 6.1 (103) | 116.4 ± 5.8 (102) | 94.9 ± 6.7 (94) |

[1] product declared 60 mg/100 mL; [2] product declared 50 mg/100 mL

for 25 μM of thymol). Higher volumes of Nafion/MWCNTs cause major increase in a background current. The presence of Nafion/MWCNTs also had an influence on the peak potential. For bare GC electrode the DPV thymol peak potential was 876 mV and for modified electrode with 10 μL Nafion/ MWCNTs the thymol peak potential was 800 mV. The negative shift of the thymol peak potential suggests catalytic effect caused by Nafion/ MWCNTs. For further work, the volume of 10 mL was used.

**Influence of preconcentration potential and time on thymol peak**

Influence of preconcentration potential and time are usually important factors on the sensitivity and detection limit of the stripping methods. In the case of thymol determination the preconcentration potential has no influence on the peak current. For further work, the -50 mV preconcentration potential was applied.

The changes in magnitude of the thymol current *vs*. preconcentration time are presented in Figure 6. The peak current increased with the increase of the preconcentration time for (a) – 2.5 μM thymol from 0.45 μA ($t_{acc}$ = 0 s) to 2.6 μA ($t_{acc}$ = 180 s), for (b) – 10 μM thymol from 1.6 μA ($t_{acc}$ = 0 s) to 6.4 μA ($t_{acc}$ = 180 s) and for (c) – 25 μM thymol from 3.6 μA ($t_{acc}$ = 0 s) to 10.6 μA ($t_{acc}$ = 180 s), respectively. For a preconcentration time higher than 60 s, practically no increase of the thymol peak current was observed. The thymol peak potential is independent on either the preconcentration potential ($E_p$ = 800 mV).

**Influence of supporting electrolyte composition**

Determination of thymol on GC electrode modified with Nafion/MWCNTs requires an acidic conditions in order to obtain a well-shaped and high peak. The best results were obtained in phosphoric acid. To improve the solubility of thymol, additionally methanol to the supporting electrolyte was added. For further measurements, the 0.1 M $H_3PO_4$ and 500 uL methanol was applied (good relation the thymol signal to the background current).

**Interferences**

The examined ions, such as: Ca(II), Mg(II) in a 100-fold excess, and Zn(II), Mn(II) in a 100-fold excess as well as Pb(II), Cd(II), Cu(II) in a 2-fold excess did not interfere. Organic compounds such as: citric acid in a 20-fold excess and glucose 25 mg/L did not interfere.

The surface-active compounds are usually a source of strong interferences in voltammetric methods. A non-ionic surface-active compound (Triton X-100) was investigated in this respect. For 0.5 mg/L of Triton X-100 concentration, no suppress of the signal was observed. Higher concentration of Triton X-100 caused suppression the signal e.g., for 2.5 mg/L of Triton X-100 by 30% and for 6 mg/L of Triton X-100 by 55%.

**Analytical performance**

The differential pulse stripping voltammetry (DP SV*)* voltammograms of thymol for the 0.2-2.2 μM concentration range and preconcentration time of 30 s are presented in Figure 7.

The detection limit obtained for short preconcentration time (15 s) was 0.1 μM with the linearity up to 5 μM (slope for regression line was 0.71 ± 0.02 [μA/μM], intercept 0.08 ± 0.04 μA, correlation coefficient 0.998). A longer preconcentration time results in a lower detection limit (for example, when the preconcentration time of 30 s was used during measurement, the detection limit was 0.06 μM. The

slope for regression line was [μA/μM]: 1.18 ± 0.02, intercept [μA]: 0.15 ± 0.06, the correlation coefficients 0.998 and the linearity was up to 3 μM.

To validate the method, the urine, dental mouthwash and liquid anti-ace were investigated.

The samples, spiked with thymol were analyzed according to the described procedure using the GC electrode modified with Nafion/MWCNTs. Determinations of thymol were performed using the standard addition method (two additions of the standard solution). Results from thymol determination are presented in Table 1. The recovery of thymol ranged from 88 to 103%. The analytical usefulness, of the presented method for the determination of thymol in samples was confirmed.

## CONCLUSIONS

The presented DPV method for the electrochemical determination of thymol using a GC electrode modified with Nafion/MWCNTs allows to determine thymol at trace levels, in concentrations as low as 0.05 μM (7.5 μg/L) for a preconcentration time of 30 s. The reproducibility of the method is good, i.e., when measured as RSD is 3.9%. Acceptable recovery (88–103%) shows that the proposed method can be used for the determination of thymol in urine, dental mouthwash and liquid anti-ace.

The preparation of GC electrode modified with Nafion/MWCNTs is very simple, short and economically acceptable. The obtained results confirm that presented method may be used into out-of-laboratory systems.

### Acknowledgments

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# VALIDATION OF LC/MS/MS METHOD FOR ASSESSMENT OF THE *IN VITRO* ACTIVITY OF SELECTED RAT CYTOCHROME P450 ISOENZYMES - APPLICATION TO EARLY DRUG METABOLISM SCREENING

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**Abstract:** A sensitive and specific liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for simultaneous determination of seven metabolites of CYP450 model substrates (acetaminophen, 4-hydroxytolbutamide, 4'-hydroxymephenytoin, 1-hydroxybufuralol, 6-hydroxychlorzoxazone, 1'- and 4'-hydroxymidazolam) in rat liver microsomes was developed. The assay used Kinetex analytical column and a gradient mobile phase consistent of acetonitrile and water with addition of 0.1% formic acid. The analysis was performed in selected reaction monitoring (SRM) mode both in positive and negative (for 6-hydroxychlorzoxazone) mode. The method was validated over the concentration ranges of 10-2000 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide, 50-2000 ng/mL for 1-hydroxybufuralol and 25-2000 ng/mL for the rest of the analytes. The intra- and inter-day precision (2-12%) and accuracy (93-119%) were within the limits set by the FDA and EMA guidelines. The developed method was successfully applied to assess the activity of selected CYP450 isoenzymes in rat liver microsomes after addition of ketoconazole.

**Keywords:** rat liver microsomes, CYP450 isoenzymes, cocktail method, LC/MS/MS, ketoconazole

The development of new drugs is determined largely by their ADME properties of which metabolism is an important factor affecting e.g., bioavailability, detoxification, drug-drug interactions and individual pharmacokinetics and pharmacodynamics variability. Drugs and xenobiotics are mainly metabolized by different oxidation and conjugation enzymes to more polar and better soluble metabolites to facilitate their excretion (1).

Cytochrome P450 system represents drug-metabolizing enzymes from a superfamily of hemoproteins, engaged in phase I metabolism. Among the CYP isoforms, family 1 to 4 are involved in metabolism of different xenobiotics, and in human more than 90% of drugs that undergo biotransformation are metabolized by CYP 1A2, 2A6, 2B6, 2C9, 2C19, 2D6, 2E1 and 3A4 (2, 3). In the development of new chemical entity, drug candidate, preclinical screening of metabolism rates and assessment of elimination routes are considered to be essential for further development.

In the field of drug discovery, a demand has risen for efficient and rapid drug metabolism screening techniques. To speed up the selection of new drug candidates, to identify the metabolic profile of new molecules, to indicate likely drug interactions or to assess the role of polymorphic enzymes, different *in vitro* systems have been developed.

LC/MS/MS technique provides a powerful solution for investigating the biotransformation of xenobiotics and is used at various stages of drug discovery and development (4). Because of the need to test a large number of compounds, recently a high-throughput screening methods were created. One of the application includes the use of the cocktail of substrates added to the microsomal incubations for simultaneous evaluation of the activities of CYP450 isoenzymes (5). Due to this approach multiple enzymes may be assessed in the same study and the effect of intra-individual variability is minimized. Recently, numerous of drug metabolism cocktails have been proposed and developed (6–15).

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According to FDA guidelines, preclinical metabolism studies of new drug candidates should be conducted using human microsomes (16), while there are numerous *in vitro* works for the assessment of preliminary metabolic pathway of new compounds using rat liver microsomes, cytosol and primary hepatocytes, because rats are the most commonly used model in the field of preclinical studies (17–20). Among the frequent examples, work of Xiao Hu et al. (21) demonstrated the variance analysis of $V_{max}$, $K_m$ and $Cl_{int}$ values from the study of enzyme kinetics of osthole and imperatorin, and showed no significant difference between these values in rat and human liver microsomes. The study demonstrated that the rat liver microsomes were reliable to analyze drug metabolism *in vitro*. The study of De Bock et al. (22) described the validation of the method for determination of metabolites of model substrates in rat microsomal matrix using earlier derivatization of metabolites. The metabolic fate of sublethal exposure of different herbicides in rat microsomes was studied in the work of Larsen et al. (23). On the basis of the results of Xue-Jin Zhao et al. (24) one may assume that from a qualitative point of view, the dog, rat and even mouse could be an appropriate species for conducting an *in vitro* study on the metabolism of quinine. However, using rodent data to estimate human risk should be aware of interspecies variability both in expression and catalytic activity of relevant CYP450 isoforms (25).

The present study aims to evaluate a simple and an accurate LC/MS/MS method for simultaneous determination of specific metabolites of six model substrates metabolized by CYP450 isoenzymes after incubation with rat liver microsomes. In the next step, the optimized method was used to establish the strength of enzyme inhibition by ketoconazole on selected CYP450 isoenzymes.

## MATERIALS AND METHODS

### Chemicals and reagents

Midazolam, 1'-hydroxymidazolam (1-OH-MZ), 4'-hydroxymidazolam (4-OH-MZ), phenacetin, acetaminophen (ACP), tolbutamide, 4-hydroxytolbutamide (4-OH-TB), bufuralol, 1-hydroxybufuralol (1-OH-BF), S-mephenytoin, 4'-hydroxymephenytoin (4-OH-MP), chlorzoxazone, 6-hydroxychlorzoxazone (6-OH-CH), dextrophan, magnesium chloride, NADPH, Folin & Ciocalteu's phenol reagent, potassium sodium tartrate tetrahydrate, cooper sulfate, sucrose, TRIS, sodium phosphate dibasic and potassium chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid was obtained from Fluka (Buchs, Switzerland). Potassium dihydrogen phosphate, sodium chloride, acetone and acetonitrile were purchased from J.T. Baker (Phillipsburg, PA, USA). Purified water (18.2 MΩ) used throughout the study was delivered by a Milli-Q water system (Millipore, Billerica MA, USA).

### Isolation of hepatic microsomes

Rat liver microsomes were prepared using a differrential centrifugation method. Liver fragments were minced with scissors, washed with TRIS/KCl (pH 7.4) buffer and homogenized. The homogenate was centrifuged (Sorval WX Ultra Series, Thermo Scientific) at approximately 11 500 × g for 20 min at 4°C. The supernatant (S9 fraction) was transferred to new centrifuge tubes and then ultracentrifuged at 100 000 × g for 60 min at 4°C. The pellet was resuspended in 0.15 M KCl with Ultra Turrax IKA T10 basic homogenizer (IKA-Werke GmbH & Co. KG Staufen, Germany) and ultracentrifuged again at 100 000 × g for 60 min at 4°C. The obtained pellet was dispersed in TRIS/sucrose buffer and stored at -80°C until use.

Protein concentration in microsomal fraction was determined by Lowry protein assay (26).

### Chromatographic conditions

Chromatographic separation was performed on a Kinetex (2.6 µm, PFP, 100 Å, 100 × 3 mm, Phenomenex, Torrance, CA, USA) analytical column using UltiMate 3000 UPLC system (Dionex, USA). The mobile phase consisted of acetonitrile containing 0.1% formic acid (eluent A) and water containing 0.1% formic acid (eluent B). At the flow rate of 500 µL/min, the amount of eluent A increased lineary from 20 to 95% in 2 min, maintained by 95% for 6 min, then returned to 20% in 3 min, and was left to equilibrate for 6 min. The total run time was 15 min.

### LC/MS/MS conditions

The analyzed compounds were detected using TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Scientific, Waltham, MA, USA). Heated electrospray ionization (H-ESI) was used both in the positive and negative (for 6-hydroxychlorzoxazone) modes. The selected reaction monitoring transitions and collision energies optimized for each metabolite and internal standard are listed in Table 1. The mass spectrometric conditions were as follows: needle voltage 4020 V, vaporizer temperature 250°C, sheath gas (nitrogen) pressure 30 psi, auxiliary gas (nitrogen) pressure 10 psi, capil-

lary temperature 370°C. The argon pressure in the collision cell was approximately 2 mTorr.

**Standard and quality control samples**

Stock solutions of metabolites of model substrates and dextrophan used as an internal standard (IS) were prepared at concentration of 0.5 mg/mL in methanol. Working solutions for calibration standards were prepared by diluting the stocks in water to the concentrations of 0.125, 0.3125, 0.625, 1.25, 3.25, 6.25, 9.375, 12.5 and 25 µg/mL of each analyte in cocktail, and 0.125, 0.375, 1.125, 10, 22.5 µg/mL for quality control samples. Dextrophan was diluted in acetone : acetonitrile mixture (1 : 1, v/v) to a final concentration of 250 ng/mL.

Calibration samples were made at 10, 25, 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide; 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for 1-hydoxybufuralol and 25, 50, 100, 250, 500, 750, 1000 and 2000 ng/mL for the rest of analyzed metabolites.

Quality control (QC) samples were prepared at 30, 800 and 1800 ng/mL for 4'-hydroxymephenytoin and 4-hydroxytolbutamide, and 90, 800 and 1800 ng/mL for other substances. Calibration standards and quality control samples were prepared by spiking 40 µL of working solution of cocktail of analytes with blank microsomal medium (without NADPH). In the next step, to stop the reaction, the mixture of acetonitrile : acetone (1 : 1, v/v) with internal standard was added. After vortex mixing and cooling down on ice, samples were centrifuged at 15 000 × g for 15 min, and supernatant was analyzed using LC/MS/MS method.

**Method validation**

Validation of the method was carried out according to the FDA and EMA bioanalytical method validation guidelines (27, 28) with respect to the linearity, selectivity, precision, accuracy, recovery, limit of detection, limit of quantification, matrix effect and post-preparative stability.

Selectivity is the ability of the analytical method for separation and quantification of the analyte in the presence of other compounds in the sample. It was assessed by studying peak interference from six independent sources of microsomes.

Calibration curves were constructed using seven to nine calibration standards and fitted by a weighted least-squares linear regression. To assess linearity, deviations of the back calculated concen-

Table 1. Selected reaction monitoring parameters for quantified analytes.

| Analyte | Q1 [m/z] | Q3 [m/z] | CE [V] |
|---|---|---|---|
| Acetaminophen | 152 | 110 | 14 |
| 4-Hydroxytolbutamide | 287 | 89 | 38 |
| 4'-Hydroxymephenytoin | 235 | 150 | 19 |
| 1-Hydroxybufuralol | 278 | 186 | 14 |
| 6–Hydroxychlorzoksazone | 180 | 120 | 20 |
| 1'-Hydroksymidazolam | 342 | 324 | 21 |
| 4'-Hydroxymidazolam | 342 | 297 | 24 |
| Dextrophan (IS) | 258 | 157 | 38 |

Table 2. Linear ranges, determination coefficients, limits of detection and limits of quantification for selected compounds.

| | ACP | 6-OH-CH | 4-OH-MP | 1-OH-BF | 4-OH-TB | 1-OH-MZ | 4-OH-MZ |
|---|---|---|---|---|---|---|---|
| Liner range [ng/mL] | 25-2000 | 25-2000 | 10-2000 | 50-2000 | 10-2000 | 25-2000 | 25-2000 |
| Determination coefficient ($r^2$) | 0.9994 | 0.9995 | 0.9916 | 0.9908 | 0.9965 | 0.9974 | 0.9983 |
| LOD [ng/mL] | 0.1 | 1 | 0.05 | 0.01 | 0.01 | 0.025 | 0.01 |
| LLOQ [ng/mL] | 25 ± 2.8 | 25 ± 2.8 | 10 ± 1.3 | 50 ± 5.9 | 10 ± 0.5 | 25 ± 3.0 | 25 ± 3.1 |

ACP - acetaminophen; 6-OH-CH - 6-hydroxychlorzoxazone; 4-OH-MP - 4'-hydroxymephenytoin; 1-OH-BF - 1-hydroxybufuralol; 4-OH-TB - 4-hydroxytolbutamide; 1-OH-MZ - 1'-hydroxymidazolam; 4-OH-MZ - 4'-hydroxymidazolam.

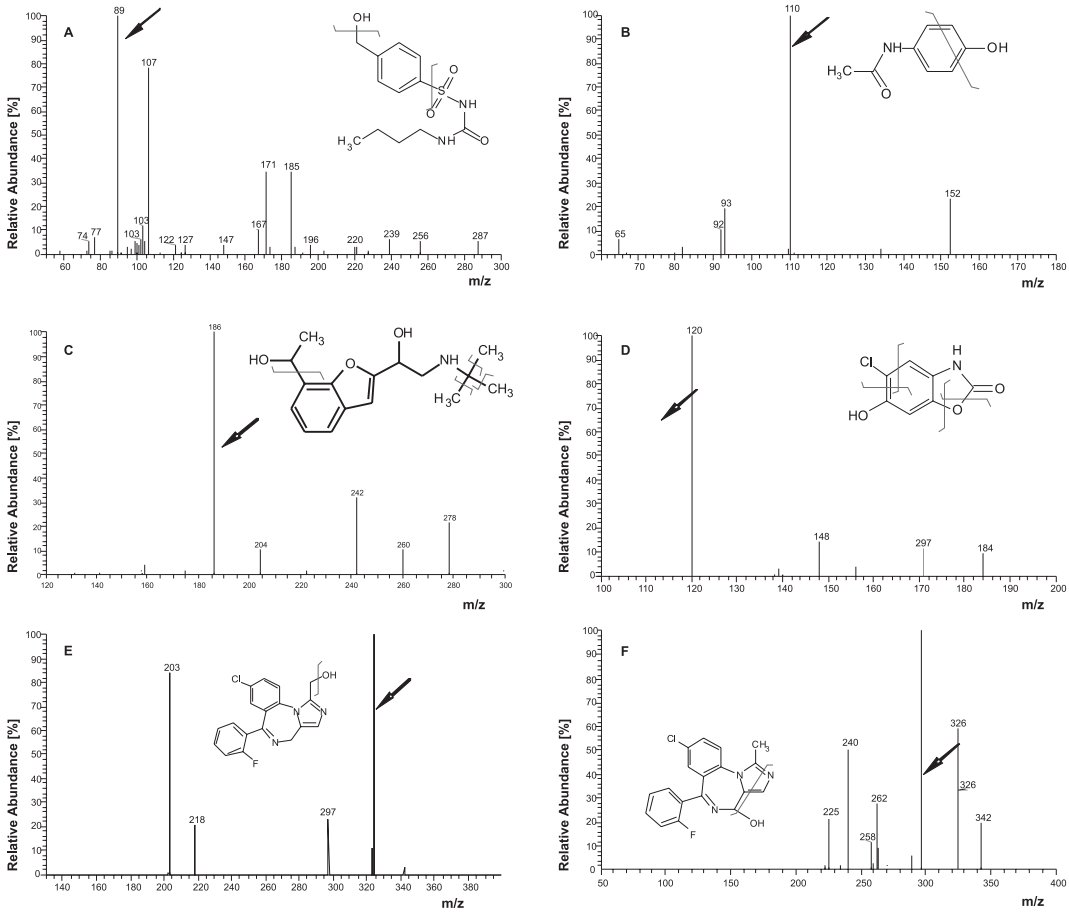


Figure 1. Fragmentation mass spectra of selected metabolites with purposed fragmentation pathways: 4-hydroxytolbutamide (A), acetaminophen (B), 1-hydroxybufuralol (C), 6-hydroxychlorzoxasone (D), 1-hydroxymidazolam (E) and 4-hydroxymidazolam (F)

trations were set at ±15% of the nominal ones, except of the LLOQ, when a deviation of ±20% was permitted.

Inter- and intra-batch precision and accuracy of the assay were verified for three concentrations of QC samples. Six replicates of sample were analyzed on the same day to determine the intra-day precision and accuracy, and on each of two separate days to determine inter-day precision and accuracy. The mean values of precision should be within 15% of the actual value, except of LLOQ, where it should not deviate more than 20%. The accuracy determined at each concentration level should not exceed 85-115%, except of LLOQ, where it should not exceed 80-120%.

The limit of detection (LOD) was defined as the concentration that generates a signal-to-noise ratio of 3, whereas the limit of quantification (LLOQ) was determined as the lowest concentration of the analytes in the sample, which can be quantified with precision lower than 20%, and an accuracy between 80% and 120%.

The stability of samples in autosampler batch was assessed by reanalyzing extracted QC samples kept under the autosampler conditions (10$^{o}$C) for 24 h. An acceptable stability was defined if the initial loss of concentration was below 15%.

Recovery and absolute matrix effect were assessed at three concentrations of QC samples by comparing the peak areas of 6 runs at each concentration for the neat solutions of analytes and the standard solutions spiked before and after protein precipitation. Recovery was calculated by dividing the mean peak area of standards spiked before extraction by mean peak area of standards spiked after extraction.

The matrix effect was evaluated by comparing the mean peak area of the analyte spiked post extraction to the mean peak of its neat solutions. The absolute matrix effect was defined as (1 - signal of post-extracted spiked sample/signal of pure solution) × 100%. The relative matrix effect was also estimated according to the work of Matuszewski (29) by assessing the variability of standard line slopes expressed as coefficient of variation. The precision of the method should not exceed 4%, and then is considered to be reliable and free from the relative matrix effect.

**Inhibition study**

To the total volume (0.5 mL) of incubation mixture of rat liver microsomes (1 mg/mL) added 0.1 M phosphate buffer (pH 7.4), 10 mM $MgCl_2$, 1 mM NADPH and substrates cocktail in concentrations near their $K_m$ values: phenacetin - 7.5 µg/mL, tolbutamide - 2.5 µg/mL, bufuralol - 12.5 µg/mL, chlorzoxazone - 15 µg/mL and midazolam - 7.5 µg/mL. To the incubation mixture ketoconazole was added in the concentration range of 0.3 to 37.5 µM. Mixtures were preincubated for 5 min at +37°C in a shaking water bath (OLS 200, Grant Instruments, Cambridge, UK). The reaction was initiated by addition of NADPH (cofactor of metabolic reaction). Following a 10 min incubation, the reaction was terminated with 500 µL of ice cold mixture of acetonitrile : acetone (1 : 1, v/v) containing dextrophan as an internal standard (250 ng/mL). Samples were subsequently cooled on ice for 20 min to precipitate the proteins, and then centrifuged at approximately 15 000 × g for 15 min at 4°C. The supernatant (200 µL) was transferred to vials, and a volume of 20 µL was injected onto the analytical column.

CYP-mediated activities in the presence of ketoconazole were expressed as a percentage of the corresponding control values. A sigmoidal shaped curve were fitted to the data and the $IC_{50}$ values for ketoconazole were calculated by fitting the Hill equation to the data using non-linear regression of the plot of percent control activity *versus* concentration of ketoconazole using Prism (GraphPad Software, Version 6). The enzymes activities in the presence of ketoconazole were compared with the control samples without addition of ketoconazole.

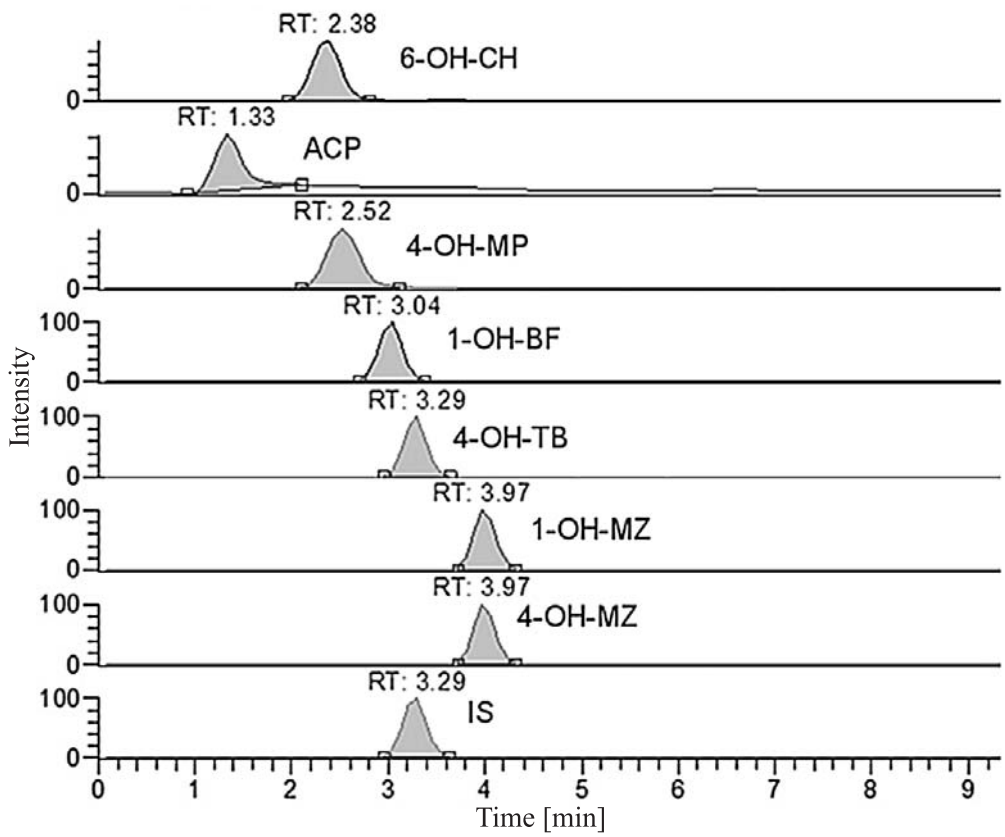


Figure 2. Extracted SRM chromatograms of simultaneous analysis of seven metabolites of model substrates and IS in calibration standard sample. Abbreviations see Table 2

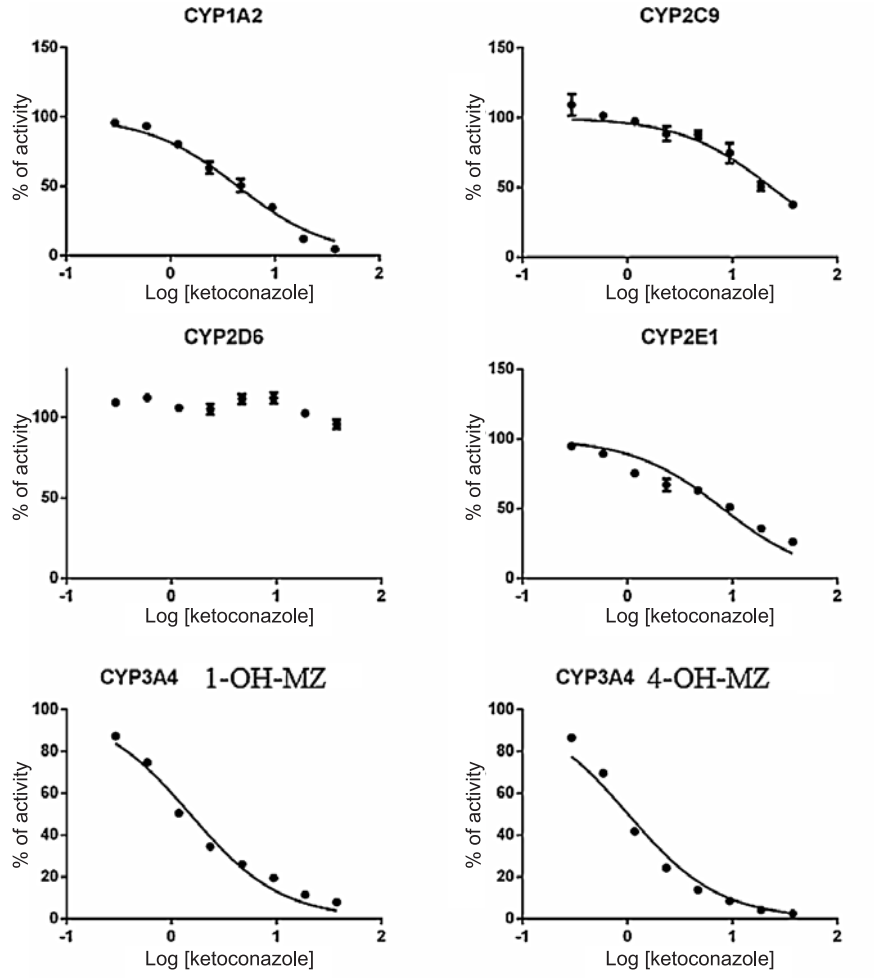


Figure 3. The activity of selected isoenzymes after incubation of model substrates of CYP450 with ketoconazole in rat liver microsomes. Data are shown as the mean ± SD, n = 3. 1-OH-MZ - 1'-hydroxymidazolam; 4-OH-MZ - 4'-hydroxymidazolam

## RESULTS AND DISCUSSION

### Method development

Metabolism studies of new compounds that occur with the participation of enzymes require simple, quick, sensitive and reproducible analytical methods. The aim of this work was to develop and validate a simple and reliable LC/MS/MS method, which can be used to determine simultaneously seven metabolites of specific substrates of selected CYP450 isoenzymes in rat liver microsomes.

Animal models are widely used to predict kinetics and toxicity of drugs in human, and cytochrome P450 enzymes are mainly involved in the metabolism of drugs both in human and rodents. The three families: CYP1, CYP2 and CYP3 play a significant role in drug biotransformation (30). Differences between e.g., human and rat enzymes concern mostly individual genes, while not only families but also subfamilies are common. Major isoenzymes of CYP450 in human liver are: CYP 1A2, CYP 2B6, CYP 2C8, CYP 2C9, CYP 2D6 and CYP 3A4/5, while in rat are: CYP 1A1, CYP 1A2, CYP 2B1, CYP 2C6, CYP 2C11, CYP 2D1/2, CYP 3A1/2 and CYP 4A1. Enzymes CYP 2C and CYP 3A appear to be the most abundant cytochromes P450 in rat and human liver, respectively (31).

Most of the described in the literature experiments deal with human microsomes, while there are a lot of work performed on rat microsomes, because rats are the most commonly used model in preclinical studies (6, 32-34). Zhang et al. (6) studied phenacetin, tolbutamide, mephenytoin, dextromethorphan and midazolam metabolism incubated

individually and in cocktail form with rat microsomes and no significant differences were observed. Phenacetin and tolbutamide metabolism in rat liver microsomes were studied by Jurica et al. (32). Coctail assay was used to study herb-drug interaction in rat microsomes by Xia et al. (33). Recently, comparison between human and rat microsomal CIME cocktail assay was verified by Videau et al. (34).

In presented work, the model substrates of selected isoenzymes of CYP450 were added in the form of cocktail to rat microsomes. Substrates were selected on the basis of a number of literature reports describing the metabolism of drugs in rats (30, 31, 35–37) and the EMA guideline describes probe substances, which can be used for evaluation of cytochrome P450 activity in human (38). The following model substrates were selected: phenacetin for CYP 1A2, tolbutamide for CYP 2C9, S-mephenytoin for CYP 2C19, bufuralol for CYP 2D6, chlorzoxazone for CYP 2E1 and midazolam for CYP 3A4. Fragmentation mass spectra of selected metabolites and proposed fragmentation pathways are shown in Figure 1.

The analytes were at first detected in full scan mass spectrum and then fragmented to product ions to ascertain their pseudo-molecular ions and to select product ions used in SRM mode. Except of chlorzoxazone and its metabolite, which responded better in negative ionization mode, all other substances were quantified in positive ion mode.

A simple protein precipitation method was used for sample purification and different precipitation mixtures were verified. The results showed that the most efficient way was precipitation using a mixture of acetonitile and acetone (1 : 1, v/v).

Table 3. Intra- and inter-day precision and accuracy of the method.

| | | ACP | 6-OH-CH | 4-OH-MP | 1-OH-BF | 4-OH-TB | 1-OH-MZ | 4-OH-MZ |
|---|---|---|---|---|---|---|---|---|
| Intra-day precision (RSD%) | LLOQ | 11.1 | 10.4 | 13.0 | 19.5 | 4.5 | 12.6 | 13.0 |
| | QC low | 3.2 | 8.8 | 4.0 | 7.5 | 4.4 | 7.1 | 5.1 |
| | QC medium | 11.0 | 7.6 | 3.4 | 2.7 | 2.5 | 1.7 | 1.0 |
| | QC high | 8.4 | 6.5 | 3.8 | 2.1 | 3.0 | 3.5 | 2.7 |
| Inter-day precision (RSD%) | LLOQ | 13.9 | 12.0 | 12.0 | 7.5 | 3.2 | 5.4 | 6.6 |
| | QC low | 5.6 | 7.5 | 7.4 | 14.9 | 6.1 | 6.3 | 4.1 |
| | QC medium | 11.7 | 5.9 | 11.0 | 4.0 | 7.5 | 4.7 | 3.0 |
| | QC high | 10.8 | 5.4 | 7.5 | 3.5 | 3.5 | 4.0 | 2.4 |
| Accuracy (%) | LLOQ | 118.7 | 106.3 | 103.1 | 83.7 | 107.0 | 97.2 | 96.5 |
| | QC low | 102.9 | 95.5 | 106.0 | 113.6 | 99.8 | 98.2 | 109.2 |
| | QC medium | 103.3 | 98.1 | 104.6 | 106.7 | 99.8 | 103.8 | 102.7 |
| | QC high | 107.4 | 93.0 | 99.7 | 99.6 | 97.6 | 105.4 | 98.2 |

Abbreviations see Table 2.

Table 4. Extraction recovery and absolute matrix effect of the method.

| | | ACP | 6-OH-CH | 4-OH-MP | 1-OH-BF | 4-OH-TB | 1-OH-MZ | 4-OH-MZ |
|---|---|---|---|---|---|---|---|---|
| Recovery [%] | QC low | 85.4 | 92.0 | 68.4 | 83.5 | 74.1 | 79.4 | 82.1 |
| | QC medium | 70.5 | 91.1 | 68.3 | 86.4 | 74.3 | 84.8 | 83.0 |
| | QC high | 75.3 | 94.8 | 71.4 | 89.0 | 78.9 | 91.1 | 90.6 |
| | Mean ± SD | 77.1 ± 7.6 | 92.6 ± 1.9 | 69.4 ± 1.8 | 86.3 ± 2.8 | 75.8 ± 2.7 | 85.1 ± 5.9 | 85.2 ± 4.7 |
| Absolute matrix effect [%] | QC low | 65.0 | 16.5 | 8.9 | -23.7 | 20.2 | 12.3 | -14.8% |
| | QC medium | 61.9 | 17.9 | 14.0 | 5.2 | 22.7 | -5.4 | 0.2 |
| | QC high | 60.1 | 10.2 | 10.1 | 4.0 | 20.8 | -6.8 | 2.0 |
| | Mean ± SD | 62.3 ± 2.5 | 14.9 ± 4.1 | 11.0 ± 2.7 | -4.8 ± 16.4 | 21.2 ± 1.3 | 0.03 ± 10.6 | -4.2 ± 1.2 |

Abbreviations see Table 2.

**Method validation**

***Selectivity and linearity***

No interference was found at the retention time of the analytes and the IS during analysis of blank microsomes from six independent sources (signal-to-noise ratio < 9). Extracted SRM chromatograms of seven metabolites of model substrates and IS in calibration standard sample are shown in Figure 2.

The assay was linear over the concentration range of 10-2000 ng/mL for 4'-hydroxymephenytoin and 1-hydroxytolbutamide, 50-2000 ng/mL for 1-hydroxybufuralol, and 25-2000 ng/mL for the rest of the analyzed metabolites. Linear ranges, determination coefficients, LOD and LLOQ values are shown in Table 2. The best linear fit of the calibration curve for each analyte was obtained using a weighting factor 1/x. Precision and accuracy of the LLOQ met the requirements both of FDA and EMA guidelines, RSD < 20% and 80-120%, respectively.

***Precission and accuracy***

For all quantified metabolites, the intra-day coefficients of variation were less than 11% for low, medium and high QC concentrations, and less than 20% for LLOQ. The inter-day data were also reproducible with CV less than 12% for prepared QC samples, and less than 14% for LLOQ. The study shows good accuracy: 93-114% for low, medium and high QC concentrations, and 83-119% for LLOQ level. Precision and accuracy data for each of the analytes are summarized in Table 3.

***Recovery and matrix effect***

Table 4 shows the results of recovery and absolute matrix effect for all selected analytes. A simple precipitation method used in this study yielded a mean recovery greater than 69% for all analytes. The extraction recovery was noticed to be consistent for each analyte over the entire QC concentration range, what can indicate that extraction efficiency of the method is reliable over the studied concentration.

The mean values of absolute matrix effect ranged from 0.03 to 62.3%, and the higher matrix effect was observed for acetaminophen (62.3%) but even so, the calibration curve covered the expected concentration range. To confirm the reliability of this method, the relative matrix effect was studied. Coefficients of variation of calibration curve slopes for compounds studied were less than 1.8% (Table 5), and according to Matuszewski indications (29), the method was free from the relative matrix effect liability.

**Stability study**

Analyzed aliquots were stable in autosampler batch in 10°C for at least 24 h. The mean values of the calculated concentrations were within 94-113% and therefore acceptable (Table 6). Consequently, extracts from sample incubation may be placed in autosampler rack for up to 24 h.

**Application of the method**

The method was used to assess the strength of inhibition of ketoconazole on selected CYP450 isoenzymes. The incubation curves are shown in Figure 3 and determined $IC_{50}$ values for ketoconazole are listed in Table 7. The results showed that ketoconazole had the most potent inhibitory effect on CYP 3A4 ($IC_{50}$ = 1 μM), but also suppressed activity of CYP 1A2 ($IC_{50}$ = 4.4 μM), CYP 2E1 ($IC_{50}$ = 8.1 μM) and CYP 2C9 ($IC_{50}$ = 23.5 μM), whereas the activity of CYP 2D6 was not changed.

Many researchers found that antifungal azoles can inhibit the activity of several cytochrome P450

Table 5. Individual and mean values of standard line slopes for analytes studied.

| | Slope of the calibration curve | | | | | | |
|---|---|---|---|---|---|---|---|
| | ACP | 6-OH-CH | 4-OH-MP | 1-OH-BF | 4-OH-TB | 1-OH-MZ | 4-OH-MZ |
| | 0.00048 | 7.8309E-06 | 0.000155 | 0.00275 | 0.00043 | 0.0046 | 0.00147 |
| | 0.00048 | 7.8867 E-06 | 0.00015 | 0.00286 | 0.00044 | 0.0049 | 0.00148 |
| | 0.0005 | 8.2371 E-06 | 0.000153 | 0.00291 | 0.00043 | 0.00478 | 0.00152 |
| | 0.00049 | 8.3464 E-06 | 0.00015 | 0.00288 | 0.00043 | 0.00468 | 0.00153 |
| | 0.00051 | 8.4954 E-06 | 0.000149 | 0.00286 | 0.00047 | 0.00461 | 0.00147 |
| | 0.00052 | 8.2371 E-06 | 0.000139 | 0.00288 | 0.00042 | 0.00491 | 0.00148 |
| Mean | 0.000496 | 8.17227 E-06 | 0.000149 | 0.00285 | 0.000436 | 0.004747 | 0.001492 |
| CV [%] | 3.3 | 3.2 | 3.7 | 1.9 | 4.0 | 2.9 | 1.8 |

Abbreviations see Table 2.

Table 6. Autosampler stability data for analytes studied.

| | Stability of analytes [%] | | | | | | |
|---|---|---|---|---|---|---|---|
| | ACP | 6-OH-CH | 4-OH-MP | 1-OH-BF | 4-OH-TB | 1-OH-MZ | 4-OH-MZ |
| QC low | 103.2 | 95.7 | 101.3 | 100.4 | 109.5 | 102.2 | 94.9 |
| QC medium | 111.6 | 106.4 | 105.7 | 106.3 | 112.6 | 105.0 | 94.4 |
| QC high | 111.2 | 102.7 | 104.7 | 103.6 | 104.4 | 105.2 | 95.7 |

Abbreviations see Table 2.

Table 7. The influence of ketoconazole on the inhibition of selected isoenzymes.

| Isoenzyme | Substrate | Metabolite | Ketoconazole $IC_{50}$ [μM] |
|---|---|---|---|
| CYP 1A2 | Phenacetin | Acetaminophen | 4.40 ± 0.29 |
| CYP 2C9 | Tolbutamide | 4-Hydroxytolbutamide | 23.5 ± 0.05 |
| CYP 2D6 | Bufuralol | 1-Hydroxybufuralol | —- |
| CYP 2E1 | Chlorzoxazone | 6-Hydroxychlorzoxazone | 8.10 ± 0.041 |
| CYP 3A4 | Midazolam | 1'-Hydroxymidazolam | 1.51 ± 0.024 |
| | Midazolam | 4'-Hydroxymidazolam | 1.02 ± 0.027 |

isoenzymes. In the work (39), Paine et al. demonstrated the inhibition of intestinal CYP 1A2 by ketoconazole. Suppression of tolbutamide hydroxylase (reaction catalyzed by CYP 2C9) by azoles in *in vitro* experiments was studied by Back et al. (40). They found that ketoconazole inhibited CYP 2C9 in human microsomes with $IC_{50}$ = 16.5 μM. The effect of non-selective cytochrome P450 inhibitors such as 1-aminobenzotriazole, SKF-525A and ketoconazole on human cytochrome P450 isoenzymes was studied by Emoto et al. (41). They found that ketoconazole exerts weak inhibitory potential on CYP 1A2 activity (Ki = 120 μM), CYP 2C competitive inhibition (Ki = 7.9 μM for CYP 2C9 and Ki = 6.9 μM for CYP 2C19), mixted inhibition on CYP 2D6 activity (Ki = 12 μM) and CYP 2E1 activity (Ki = 41 μM) and competitive inhibition on CYP 3A4 (Ki = 0.02 μM).

The influence of ketoconazole on amiodarone metabolism both in human and rat microsomes was studied by Elsherbiny et al. (42). Interestingly, in rat liver microsomes ketoconazole exerted higher inhibitory potential on CYP 2D enzymes than on CYP 3A family. The human isoenzymes most inhibited by ketoconazole were CYP 1A1, CYP 3A4, CYP 2D6 and CYP 1A2, respectively. The working groups of Kobayashi et al. (43) and Eagling et al. (44) studied the effect of different inhibitors on cytochrome P450 activity in rats and showed that ketoconazole is not specific CYP 3A inhibitor, but it can suppress the activity of other isoenzymes. Only in one *in vivo* work, it was shown that ketoconazole had no effect on cytochrome P450 activity in rats (45).

Despite the use in presented studies different substrates and different substrate concentrations it can be seen that ketoconazole has the strongest inhibitory potential on CYP3A4 but this drug is also strong inhibitor of CYP1A2 and CYP2E1 and moderate inhibitor of CYP2C9. Presented data are generally in good agreement with earlier cited studies and confirmed that ketoconazole should not be consider as a selective CYP 3A4 inhibitor in rat liver microsomes.

## CONCLUSIONS

A sensitive and an accurate LC/MS/MS method has been developed for the simultaneous evaluation of the activities of six cytochrome P450 izoenzymes in rat liver microsomes. The method was successfully validated following FDA and EMA guidelines that ensures the acceptability of its performance and the reliability of analytical results. The applied screening cocktail was verified with ketoconazole, a known CYP450 enzyme inhibitor. The resulting method can be used as a first and rapid screening of new compounds, drug candidates using rat liver microsomes.

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**Declaration of interest**

The authors report no conflict and declaration of interest.

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# DETERMINATION AND CHARACTERIZATION OF SELECTED FLUOROQUINOLONES OXIDATION PRODUCTS UNDER POTASSIUM PERMANGANATE TREATMENT

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**Abstract:** A simple, sensitive and reproducible ultra-performance liquid chromatography (UPLC) method for the determination of: danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin and pefloxacin oxidation stability under permanganate treatment in acidic conditions at pH from 3.0 to 6.0, was developed. Chromatographic separations were carried out using the Acquity UPLC BEH $C_{18}$ column; (2.1 × 100 mm, 1.7 µm particle size). The column was maintained at 40°C, and eluted under gradient conditions using from 100% to 75% of eluent A over 10 min for danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin or 95% to 75% of eluent A over 10 min for pefloxacin, at a flow rate of 0.3 mL/min. Eluent A: 1% (v/v) formic acid in water; eluent B: 0.1% (v/v) formic acid in acetonitrile. Satisfactory resolution was obtained for oxidation products. The correlation coefficients and determination coefficients ($R^2$) obtained for linear model for all examined fluoroquinolones were greater than 0.99. Linearity range was observed in the concentration range 0.06–0.13 mg/mL. Sensitivity of the method was good. The LOD and LOQ values were found to be 0.01 mg/mL and from 0.02 to 0.04 mg/mL, respectively. Good precision and intermediate precision with %RSD less than 2.0% was observed. The oxidation processes followed kinetic of the second order reaction and depended upon solution acidity. Oxidation of fluoroquinolones proceeded at piperazine moiety yielding respective hydroxy and oxo analogs, and leaving the quinolone fragment intact. Structures of products formed were assigned on a basis of UPLC/MS/MS fragmentation pathways.

**Keywords:** fluoroquinolones, oxidation, kinetic evaluation, UPLC-MS/MS

Fluoroquinolones are antimicrobial agents widely used in human and veterinary medicine due to their broad activity spectrum against Gram-positive and Gram-negative bacteria and good oral intake properties. After administration, fluoroquinolones are only partially metabolized in the body and largely excreted in their pharmacologically active forms (1, 2).

In stability studies of pharmaceuticals, beside hydrolysis in acidic and basic solutions and verification of photostability, evaluation of an influence of oxidizing agents has been recommended (3). It is worth noting, that the oxidation process of drugs, especially antibiotics, has focused more attention as an arising issue in the environmental protection.

The literature survey evidenced results of oxidation of ciprofloxacin, enrofloxacin, lomefloxacin, norfloxacin, ofloxacin, pipemidic acid and flumequine using $MnO_2$ followed by evaluation of the reaction kinetics and analysis of chemical structure of degradation products formed (4, 5).

Moxifloxacin, as well as ciprofloxacin, difloxacin, lomefloxacin, norfloxacin and ofloxacin and theirs oxidation products during reaction with $KMnO_4$ in acidic solution of pH ranging from 3.0 to 6.0 were determined by UPLC-MS/MS. Mechanism of the process, the kinetic analysis and structures of oxidation products were presented in our recent publications (6, 7).

Moreover, an advanced oxidation technologies, notably ozonation of thirteen fluoroquinolone, including danofloxacin, enrofloxacin, pefloxacin and sparfloxacin with a secondary wastewater effluent matrix was also reported (8).

Similar studies were carried out for ciprofloxacin, enrofloxacin and flumequine in phosphate buffer solutions at pH values ranging from 3.0 to 9.0. Moiety-specific reaction kinetics were meas-

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ured and elimination of biological activity during aqueous ozonation processes was investigated (9, 10).

Another advanced oxidation process, electron pulse radiolysis, was studied for chemical degradation of danofloxacin, enrofloxacin, marbofloxacin, orbifloxacin and flumequine in buffer solution adjusted to pH 7.0. Kinetics and degradation mechanism were investigated. In addition, the products of γ irradiation degradation of fluoroquinolones were analyzed by LC-MS to elucidate the probable pathways of advanced oxidation/reduction processes (AO/RPs) degradation (11).

Liu et al. studied the degradation products of danofloxacin under stressed conditions (hydrolysis, oxidation and photolysis). The oxidative study was carried out in 30% $H_2O_2$ at room temperature for 12 h. Seven degradation products were detected using hybrid ion trap/time-of-flight mass spectrometry (LC/MS-IT-TOF) (12).

Other advanced oxidation processes, catalytic wet air oxidation, anodic oxidation with electrogenerated $H_2O_2$, electro-Fenton, photoelectron-Fenton and solar photoelectron-Fenton, oxidation with $H_2O_2$ in the presence of copper oxide, titanium carbide and silicon nitride nanoparticles as well as oxidation by chlorine dioxide and conductive-diamond electrochemical oxidation, were studied for the chemical degradation of enrofloxacin in aqueous solution (13-17). The primary degradation products formed during the process have been identified (13-15).

Recently our interest focused on UPLC coupled with mass spectrometry technique for the separation and identification of oxidation products since the efficiency, sensitivity and run time became an important factor in the pharmaceutical analysis.

Herein, we report on the development of a new UPLC-MS/MS method for the determination of danofloxacin (DAN), enrofloxacin (ENR), marbofloxacin (MAR), orbifloxacin (ORB), pefloxacin (PEF) and their oxidation products during reaction with $KMnO_4$ in solutions of different pH ranging from 3.0 to 6.0. The method was used for kinetic studies and identification of obtained degradation products of examined fluoroquinolones.

Potassium permanganate is widely used as an oxidizing agent in synthetic as well as in analytical chemistry and also as a disinfectant. Permanganate Mn(VII) is the most potent oxidation state in alkaline as well as in acid medium. The oxidation by permanganate ion finds extensive application in organic syntheses, especially since the advent of phase transfer catalysis. Kinetic studies are important sources of mechanistic information on the reaction.

The presence and accumulation of fluoroquinolones in aquatic environments even at low concentrations may pose threats to the ecosystem and human health by inducing increase and spread of bacteria drug-resistance due to long-term exposure. Chemical oxidation using permanganate has been widely used for treatment of pollutants in drinking water and wastewater applications for over 50 years (18, 19).

In view of potential pharmaceutical importance of DAN, ENR, MAR, ORB and PEF and lack of the literature on this type of oxidation of examined drugs and the complexity of the reaction, a detailed study of the reaction becomes important. Our study deals with the title reaction to investigate the redox chemistry of permanganate in solutions of different pH and to arrive at a suitable mechanism for oxidation of DAN, ENR, MAR, ORB and PEF by permanganate on the basis of kinetic results. It seems, that oxidation of model fluoroquinolones under $KMnO_4$ in acidic medium, opens up the possibility for application in drug stability studies and environmental protection studies in the process of utilization of drug traces.

## EXPERIMENTAL

### Chemicals and reagents

Danofloxacin Cat. No. 33700-100MG-R Sigma-Aldrich (Germany), Enrofloxacin Cat. No. OR6-237 Ranbaxy (Poland), Marbofloxacin CRS, European Pharmacopoeia Reference Standard, Council of Europe – EDQM CS 30026 F-67081 (France, Strasbourg, Cedex), Orbifloxacin Cat. No. 34041-100MG-R Sigma-Aldrich (Germany), Pefloxacin mesylate dihydrate Cat. No. P0106-10G Sigma-Aldrich (Germany). HPLC grade methanol, acetonitrile and formic acid (98%) were purchased from J.T. Baker (Netherlands). HPLC grade water was obtained from HLP 5 (HYDROLAB Poland) apparatus and was filtered through 0.2 μm filter before use.

### Standard solution

The amount of 0.2 g of all fluoroquinolones was weighed with a precision of 0.1 mg. PEF was dissolved in the volume of 50 mL of methanol, and filled up to 100 mL with the same solvent. The sample weight of DAN and ORB was dissolved in the volume of 5 mL of glacial acetic acid, and filled up to 100 mL with methanol. The sample weight of ENR was dissolved in 48 mL of methanol with the addition of 2 mL of chloroform, and filled up to 100 mL with the same solvent. Solution of MAR was

prepared by dissolving the amount of 0.2 g of MAR in the volume of 50 mL of methanol-water mixture (1 : 1, v/v), and filled up to 100 mL with the same solvent. For method validation, solutions containing different concentrations of the examined fluoroquinolones in the range 0.06–0.13 mg/mL were prepared.

**Oxidation study of the drug substance**

The amount of 0.5 mL of methanol solutions of DAN, ENR, MAR, ORB, PEF (2.0 mg/mL), 2.5 mL demineralized water, 5.0 mL ammonium acetate buffer solution prepared according to European Pharmacopeia (16) with proper pH (3.0, 4.0, 4.5, 5.0 or 6.0) and 2 mL 0.002 M $KMnO_4$ was added to 10.0 mL flasks. The test solutions were incubated at room temperature and 1 µL of each reaction mixture was injected onto ACQUITY UPLC system after 15, 30, 45 and 60 min, respectively. Before the measurements of test samples, the analysis of solutions containing identical components as test samples but without $KMnO_4$ was done. The analyses were performed in triplicate.

**UPLC/MS/MS analysis**

The UPLC-MS/MS system consisted of a Waters ACQUITY® UPLC® (Waters Corporation, Milford, MA, USA) coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). Chromatographic separations were carried out using the Acquity UPLC BEH (bridged ethyl hybrid) $C_{18}$ column; 2.1 × 100 mm, and 1.7 µm particle size. The column was maintained at 40°C, and eluted under gradient conditions using from 100% to 75% of eluent A over 10 min for DAN, ENR MAR, ORB or 95% to 75% of eluent A over 10 min for PEF, at a flow rate of 0.3 mL/min. Eluent A: 1% (v/v) formic acid in water; eluent B: 0.1% (v/v) formic acid in acetonitrile.

Chromatograms were recorded using Waters eλ PDA detector. Compound concentration (%*i*) after oxidation induced by $KMnO_4$ was calculated from quotient of peak area (A*i*) to the sum of all peaks areas (Σ*A*) on chromatograms according to formulation $\%i = (Ai/\Sigma A)100$ at $\lambda = 294$ nm. Spectra were analyzed in 200-700 nm range with 1.2 nm resolution and sampling rate 20 points/s.

MS detection settings of Waters TQD mass spectrometer were as follows: source temperature 150°C, desolvation temperature 350°C, desolvation gas flow rate 600 L/h, cone gas flow 100 L/h, capillary potential 3.00 kV, cone potential 20 V. Nitrogen was used for both nebulizing and drying gas. The data were obtained in a scan mode ranging from 50 to 1000 m/z in time 0.5 s intervals; 8 scans were summed up to get the final spectrum.

Collision activated dissociations (CAD) analyses were carried out with the energy of 30 eV, and all the fragmentations were observed in the source. Consequently, the ion spectra were obtained by scanning from 50 to 500 m/z range. Data acquisition software was MassLynx V 4.1 (Waters).

**Method validation**

The described method was validated for the determination of DAN, ENR, MAR, ORB and PEF in the presence of oxidation products by UPLC method according to ICH guidelines (20, 21).

***Specificity***

To demonstrate the specificity of the developed UPLC method the solutions of DAN, ENR, MAR, ORB, PEF after oxidation stress were analyzed. Oxidation study was performed in 0.002 M $KMnO_4$ solution in ammonium acetate buffer at pH 4.0; the solution was left for 15 min at room temperature.

***System suitability***

The system suitability parameters were defined with respect to tailing factor and resolution of examined fluoroquinolones peaks using solutions of DAN, ENR, MAR, ORB, PEF, after oxidation stress at pH 4.0.

***Linearity***

The linearity for DAN, ENR, MAR, ORB, PEF was assessed by injecting six separately prepared solutions covering the range of 0.06–0.13 mg/mL. The slope of regression line, y-intercept, standard deviation of slope and intercept, correlation coefficient, $R^2$ value and standard error of residuals of the calibration curve were calculated using the program Statistica v. 10. Next, to determine whether the residuals have normal distribution, the Shapiro-Wilk statistical test was used.

***Limit of detection (LOD) and limit of quantification (LOQ)***

Based on the standard error of residuals (Se) and the slope (a) of the calibration plots and following the formula LOD = 3.3Se/a and LOQ = 10Se/a, the LOD and LOQ values for chosen fluoroquinolones were estimated.

***Precision***

The repeatability of the method was checked by a sixfold analysis of the concentration level 0.10

**Table 1.** Validation of the method.

| Parameter | DAN | ENR | MAR | ORB | PEF |
|---|---|---|---|---|---|
| $t_R$ (min)[a] | 7.19 ± 0.05 | 7.49 ± 0.03 | 6.21 ± 0.02 | 7.64 ± 0.06 | 5.37 ± 0.04 |
| Resolution[b] | 6.00 | 5.50 | 1.33 | 6.40 | 7.00 |
| LOD (mg/mL) | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| LOQ (mg/mL) | 0.03 | 0.02 | 0.02 | 0.04 | 0.03 |
| Linear range (mg/mL) | 0.06 – 0.12 | 0.07 – 0.13 | 0.06 – 0.12 | 0.06 – 0.13 | 0.06 – 0.13 |
| Regression equation (y): | | | | | |
| Slope ($a \pm S_a$) | 189794.7 ± 5336.2 | 210743.2 ± 4944.8 | 346248.3 ± 6511.1 | 35454.2 ± 12962.1 | 116615.0 ± 3335.4 |
| Intercept ($b \pm S_b$) | –183.2 ± 491.5 | –1160.2 ± 526.3 | –1245.4 ± 582.4.5 | –1453.7 ± 1287.9 | –200.5 ± 331.4 |
| $t = b/S_b$ | $-0.37 < t_{\alpha,f}$ statistically insignificant | $-2.20 < t_{\alpha,f}$ statistically insignificant | $-2.14 < t_{\alpha,f}$ statistically insignificant | $-1.13 < t_{\alpha,f}$ statistically insignificant | $-0.61 < t_{\alpha,f}$ statistically insignificant |
| Shapiro-Wilk test for residuals | 0.8747 (p = 0.07) | 0.9568 (p = 0.75) | 0.9157 (p = 0.32) | 0.9273 (p = 0.35) | 0.9128 (p = 0.23) |
| Correlation coefficient | 0.9996 | 0.9978 | 0.9986 | 0.9934 | 0.9959 |
| $R^2$ value | 0.9989 | 0.9951 | 0.9968 | 0.9855 | 0.9911 |
| Precision (% RSD) | 0.56 | 0.89 | 0.51 | 0.82 | 0.48 |
| Intermediate precision (% RSD) | 1.10 | 1.21 | 0.93 | 1.18 | 1.01 |

$t_{\alpha,f} = 2.306$ critical value of Student's t-test for degrees of freedom f = 8 and significance level $\alpha = 0.05$. [a] Mean ± SD (n = 6), [b] The mean values for n = 6. Resolutions were calculated between two adjacent peaks.

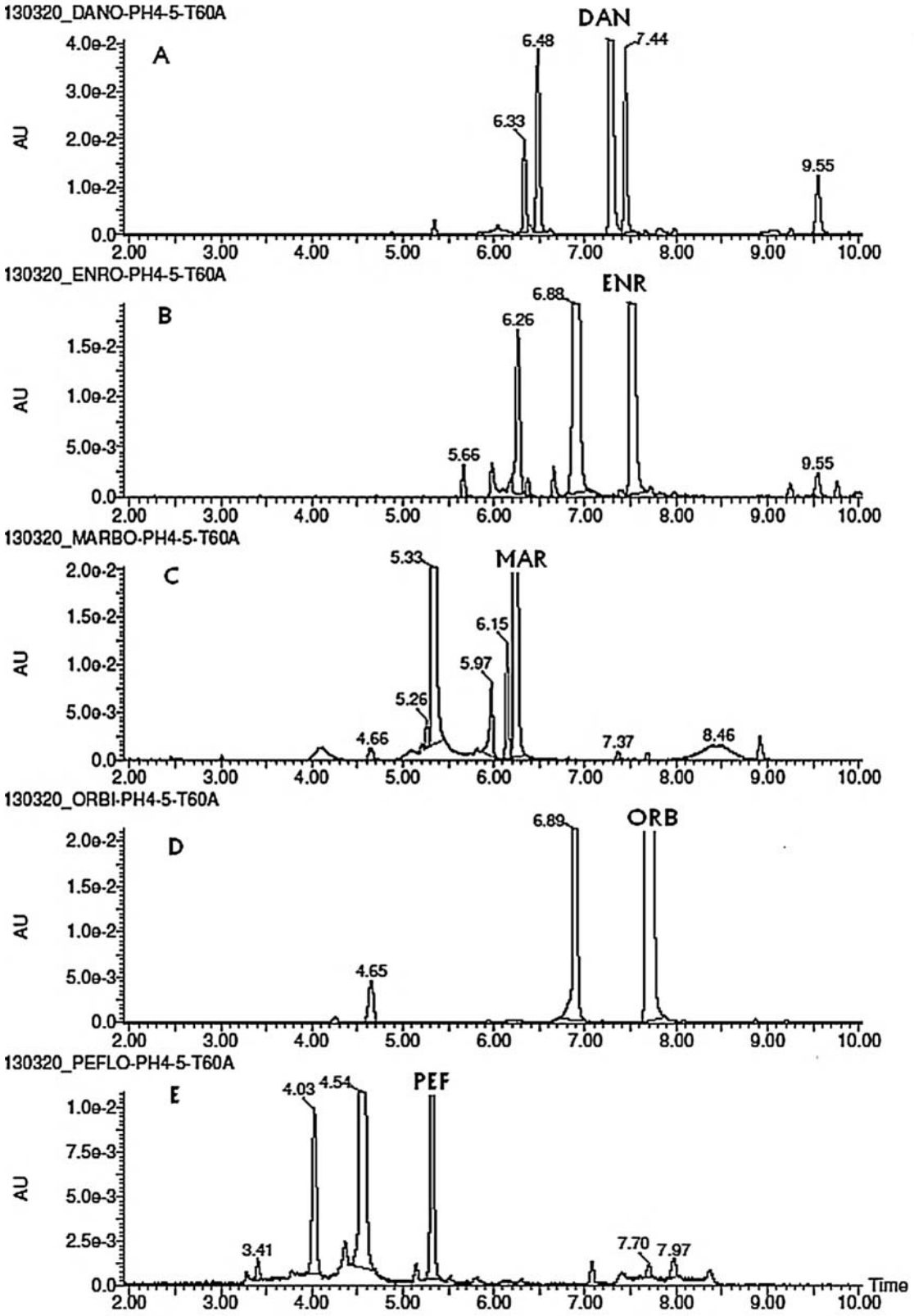



Figure 1. UPLC chromatograms registered for fluoroquinolones after oxidation induced by $KMnO_4$ at pH = 4.5: A, DAN $t_R$ = 7.24; B, ENR $t_R$ = 7.50; C, MAR $t_R$ = 6.23; D, ORB $t_R$ = 7.68; E, PEF $t_R$ = 5.33

mg/mL of DAN, ENR, MAR, ORB, PEF solutions. The same protocol was followed for three different days to study the intermediate precision of the proposed method. Different analysts prepared different solutions on different days. The RSD (%) of the peak area of examined fluoroquinolones was calculated.

***Robustness***

To demonstrate the robustness of the method deliberate small changes of flow rate, content of acetonitrile and column temperature were made around the optimal values. The mobile phase flow rate was 0.30 mL/min; to study the effect of the flow rate on resolution, the flow rate was changed to 0.27 and 0.33 mL/min. The effect of the column temperature was studied at 36°C and 44°C (instead of 40°C), and the mobile phase composition was changed +5% from the initial composition.

## RESULTS AND DISCUSSION

Apart from hydrolysis and photostability assays, an influence of oxidizing agents in the process of forced degradation is an integral part of

Table 2. Oxidation reaction rate constant of fluoroquinolones in solution at different pH.

| Component | pH | Rate constant k [$min^{-1}$] | Correlation coefficient r |
|---|---|---|---|
| PEF | 3.0 | $3.00 \times 10^{-4}$ | 0.9908 |
| | 4.0 | $11.00 \times 10^{-4}$ | 0.9596 |
| | 5.0 | $11.00 \times 10^{-4}$ | 0.9760 |
| | 6.0 | $3.00 \times 10^{-4}$ | 0.9994 |
| MAR | 3.0 | $3.00 \times 10^{-4}$ | 0.9531 |
| | 4.0 | $3.00 \times 10^{-4}$ | 0.9852 |
| | 5.0 | $3.00 \times 10^{-4}$ | 0.9892 |
| | 6.0 | $1.00 \times 10^{-4}$ | 0.9833 |
| ENR | 3.0 | $0.65 \times 10^{-4}$ | 0.9998 |
| | 4.0 | $2.00 \times 10^{-4}$ | 0.9967 |
| | 5.0 | $2.00 \times 10^{-4}$ | 0.9955 |
| | 6.0 | $0.98 \times 10^{-4}$ | 0.9837 |
| DAN | 3.0 | $2.00 \times 10^{-4}$ | 0.9242 |
| | 4.0 | $1.00 \times 10^{-4}$ | 0.8537 |
| | 5.0 | $2.00 \times 10^{-4}$ | 0.8771 |
| | 6.0 | $1.00 \times 10^{-4}$ | 0.8303 |
| ORB | 3.0 | $0.35 \times 10^{-4}$ | 0.9949 |
| | 4.0 | $0.32 \times 10^{-4}$ | 0.9678 |
| | 5.0 | $0.27 \times 10^{-4}$ | 0.9806 |
| | 6.0 | $0.22 \times 10^{-4}$ | 0.9774 |

Table 3. The kinetic results of investigated fluoroquinolones under $KMnO_4$ oxidation at pH 4.5 at room temperature.

| Component | Rate constant k [$min^{-1}$] | $t_{0.1}$ [min] | $t_{0.5}$ [min] | Correlation coefficient r |
|---|---|---|---|---|
| DAN | $2.00 \times 10^{-4}$ | 5.56 | 50.00 | 0.9169 |
| ENR | $2.00 \times 10^{-4}$ | 5.56 | 50.00 | 0.9788 |
| MAR | $3.00 \times 10^{-4}$ | 3.70 | 33.33 | 0.9937 |
| ORB | $0.25 \times 10^{-4}$ | 44.44 | 400.00 | 0.9709 |
| PEF | $10.00 \times 10^{-4}$ | 1.11 | 10.00 | 0.9654 |

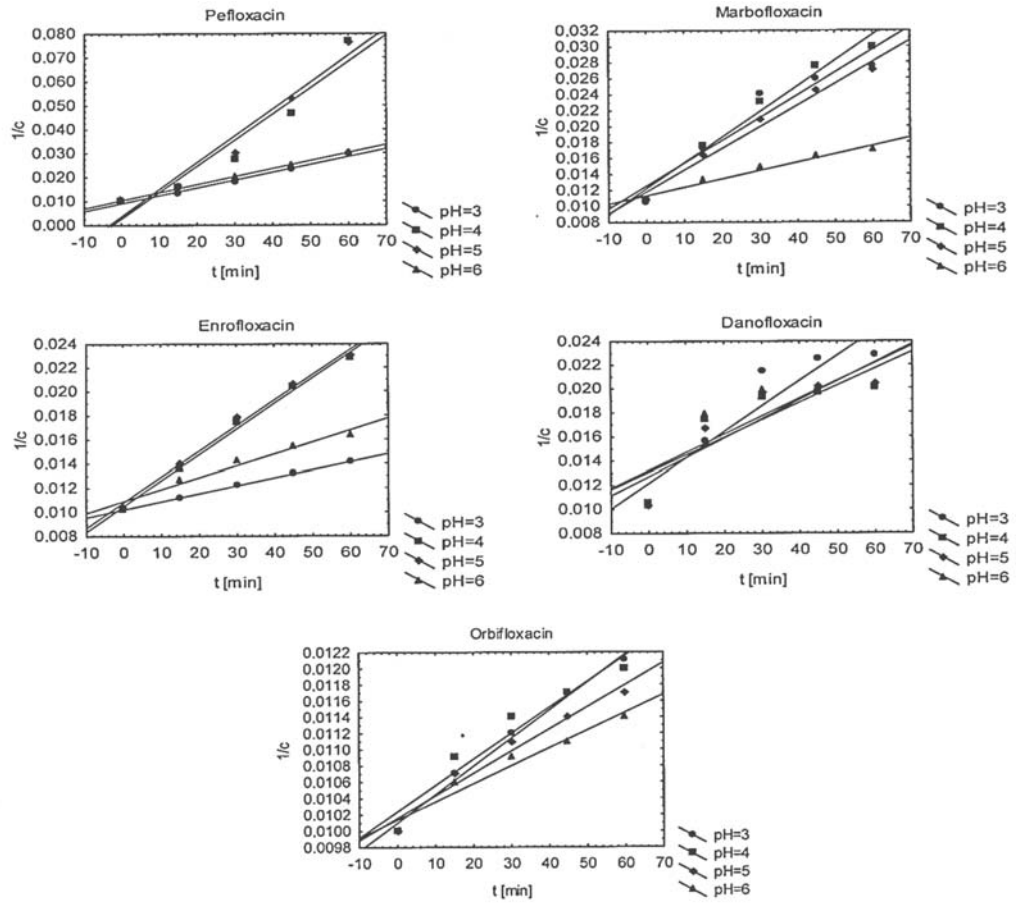



Figure 2. The 1/c = f(t) graph of oxidation of DAN, ENR, MAR, ORB and PEF induced by $KMnO_4$ in acidic medium

stress studies used for the stability evaluation of pharmaceutical products and should be checked (3). This effect may be evaluated by using validated analytical procedures enabling the determination of decreasing concentration of examined substance and detection of degradation products.

**Optimization of chromatographic conditions**

The main target of the chromatographic method was to achieve the separation of oxidation products and the main components DAN, ENR, MAR, ORB and PEF. To optimize the chromatographic separation, preliminary experiments were performed to test mobile phases containing different mixtures of acetonitrile and water (50/50, 83/17, 75/25, v/v); always the same amount of mobile phase additive was used. The solutions of determined fluoroquinolones after oxidation stress (buffer solution at pH = 4.0, 30 min incubation) was analyzed. We took advantage of a mixture acetonitrile/water 83/17 (v/v), with 0.1% of formic acid to obtain good peak resolution and symmetry.

**Method validation**

The developed UPLC method was specific to examined fluoroquinolones and guaranteed obtaining well shaped peaks both for active substances and coexisting oxidation products. Peaks of main components were well resolved from oxidation products in chromatograms and no interference that could have an influence on the obtained results was possible (Table 1). The main peak purity was examined with MS spectra using CODA algorithm (Waters Corporation, Milford, MA, USA). The investigated MS spectra uniquely contained signals corresponding to the chosen fluoroquinolones and solvent.

Satisfactory resolution was also obtained for oxidation products, peaks appearing in chromatograms were sufficiently well resolved and could be analyzed by mass spectrometry (Fig. 1).

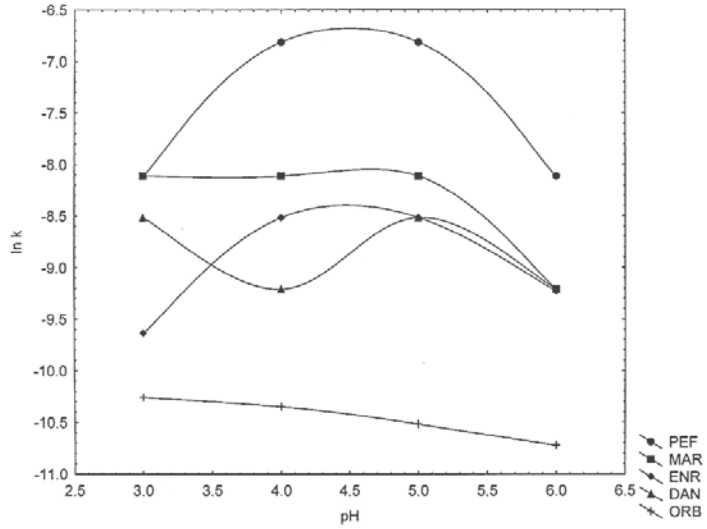
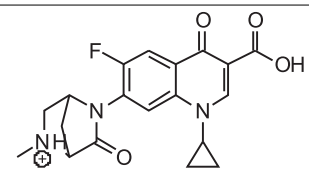
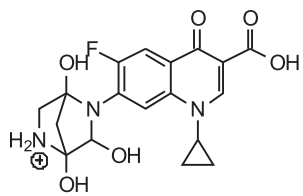
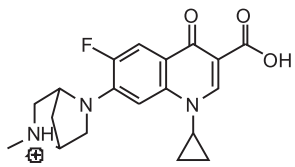
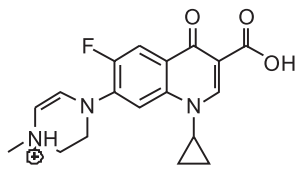
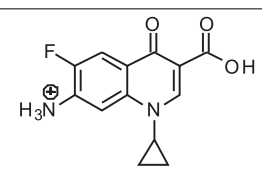



Figure 3. The ln k = f (pH) graph of oxidation of DAN, ENR, MAR, ORB and PEF induced by $KMnO_4$

Table 4. Products of oxidation of DAN induced by $KMnO_4$ in acidic conditions.

| Product | RT [min] | $[M + H]^+$ | Fragmentation ions | Proposed structure |
|---|---|---|---|---|
| **DP-1** | 6.33 | 372.1 | 354.1, 328.2, 300.2, 284.1, 257.1, 245.1, 219.1 |  |
| **DP-2** | 6.48 | 392.1 | 374.1, 356.1, 330.1, 312.1, 269.1, 257.1, 245.1, 219.1 |  |
| **DAN** | 7.24 | 358.2 | 340.2, 314.2, 283.1, 271.1, 269.1, 257.1, 255.1, 245.1, 231.1, 229.1, 219.1 |  |
| **DP-3** | 7.44 | 344.1 | 326.1, 300.2, 284.1, 257.1, 245.1, 219.1 |  |
| **DP-4** | 9.55 | 263.1 | 245.1, 219.1, 205.0 |  |

Regression analysis results obtained for examined fluoroquinolones are presented in Table 1. The correlation coefficients and determination coefficients ($R^2$) obtained for linear model for all determined fluoroquinolones were greater than 0.99. The y-intercepts of the linear equation for DAN, ENR, MAR, ORB and PEF were statistically insignificant. The distribution of the residuals can well be approximated with a normal distribution as it is shown by p-values ($p > 0.05$) of the Shapiro-Wilk normality test. Based on regression analysis, it was assumed that the calibration data fitted well to linear model. Linearity range, was observed in the concentration range 0.06–0.13 mg/mL for examined fluoroquinolones.

Sensitivity of the method was good. The LOD and LOQ values were found to be 0.01 mg/mL and from 0.02 to 0.04 mg/mL, respectively. Good precision and intermediate precision with %RSD less than 2.0% was observed. Detailed results are presented in Table 1. In all the deliberately varied chro-

Table 5. Products of oxidation of ENR induced by $KMnO_4$ in acidic conditions.

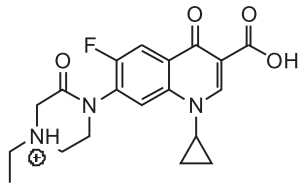
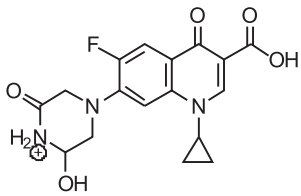
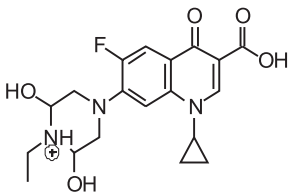
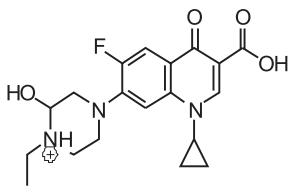
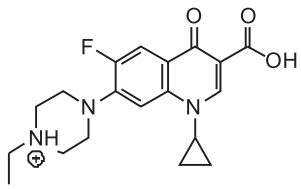
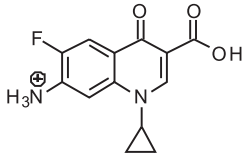
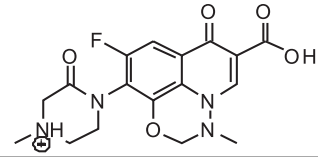
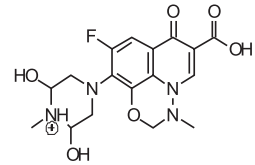
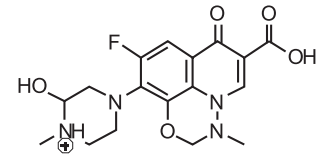
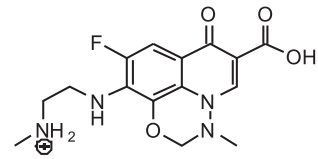
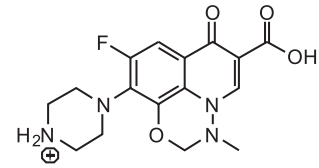
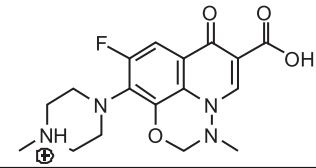
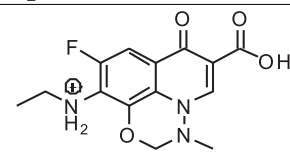
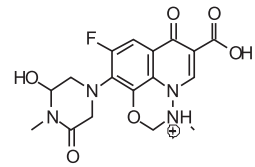
| Product | RT [min] | $[M + H]^+$ | Fragmentation ions | Proposed structure |
|---|---|---|---|---|
| **EP-1** | 5.66 | 374.2 | 356.1, 346.1, 330.2, 328.1, 302.1, 287.1, 259.1, 245.1, 219.1 |  |
| **EP-2** | 6.26 | 362.1 | 362.1, 344.1, 318.1, 290.1, 272.1, 245.1, 217.1, 191.1 |  |
| **EP-3** | 6.37 | 392.2 | 374.2, 356.1, 330.2, 312.2, 284.1, 271.1, 245.1 |  |
| **EP-4** | 6.88 | 376.2 | 358.2, 314.2, 286.1, 258.1, 230.1 |  |
| **ENR** | 7.45 | 360.2 | 342.2, 316.2, 288.2, 286.2, 260.1, 258.1, 245.1, 217.1, 205.1 |  |
| **EP-5** | 9.55 | 263.1 | 245.1, 219.1, 205.0, 179.1 |  |

Table 6. Products of oxidation of MAR induced by $KMnO_4$ in acidic conditions.

| Product | RT [min] | $[M + H]^+$ | Fragmentation ions | Proposed structure |
|---|---|---|---|---|
| **MP-1** | 4.66 | 377.1 | 359.1, 349.1, 333.1, 305.1, 289.1, 262.1, 246.1, 218.1, 192.1 |  |
| **MP-2** | 5.26 | 395.1 | 377.1, 351.2, 337.1, 333.1, 315.1, 309.1, 279.1, 262.1, 236.1, 218.1 |  |
| **MP-3** | 5.33 | 379.1 | 361.1, 343.1, 317.1, 275.1, 247.1, 233.1, 194.1 |  |
| **MP-4** | 5.97 | 337.1 | 319.1, 303.1, 293.1, 264.1, 262.1, 250.1, 234.1, 206.1 |  |
| **MP-5** | 6.15 | 349.1 | 331.1, 305.1, 288.1, 231.1, 191.1 |  |
| **MAR** | 6.19 | 363.2 | 345.1, 319.2, 276.1, 260.1, 248.1, 234.1, 219.1, 205.1, 191.1, 163.1 |  |
| **MP-6** | 7.37 | 308.1 | 290.1, 276.1, 250.1, 222.1, 207.1, 194.1 |  |
| **MP-7** | 8.46 | 393.1 | 375.1, 289.1, 275.1, 218.1, 151.1 |  |

matographic conditions (flow rate, column temperature, mobile phase composition), chosen fluoroquinolones and degradation products were adequately resolved, and the order of elution remained unchanged.

**Oxidation of examined fluoroquinolones by $KMnO_4$ in acidic medium**

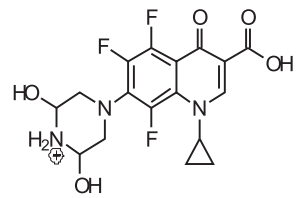
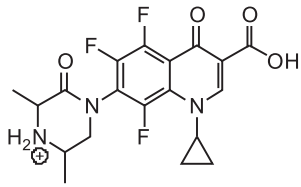
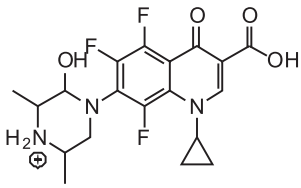
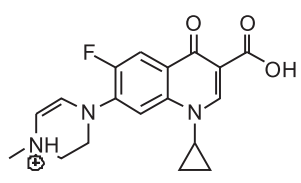
The effect of $KMnO_4$ on the oxidation of DAN, ENR, MAR, ORB and PEF has been tested in solutions of pH 3.0 to 6.0. It was found that the oxida-

tion process is dependent on the pH of the solution, incubation time and the type of fluoroquinolone. Immediately after mixing the solutions, only a single peak of fluoroquinolones deriving from starting materials were observed in chromatograms of the tested solutions. During the oxidation process, additional peaks appeared in chromatograms, whose area increased with increasing reaction time. After 60 min of incubation, the ORB had three oxidation products (OP-1 - OP-3), DAN four oxidation products (DP-1 - DP-4), and in the case of ENR and PEF five oxidation products were observed (EP-1 - EP-5 and PP-1 - PP-5). The highest number of the oxidation products was obtained for MAR ( MP-1 - MP-7). Some chosen chromatograms presented in Figure 1 show the degradation profile of DAN, ENR, MAR, PEF and ORB. Degradation process of tested fluoroquinolones increased with increasing acidity of the solution in the pH range from 6.0 to 3.0, generally reaching the highest values at pH 4.5. The percentage of degradation products in the final stage of oxidation after 60 min was as follows: 48.92% for DAN, 59.51% for ENR, 62.74% for MAR, 13.5% for ORB and 85.33% for PEF.

**Kinetic evaluation**

The analysis of the equation $1 / c = f ( t)$ for the oxidation of DAN, ENR, MAR, ORB and PEF in the pH range 3.0-6.0 demonstrated that oxidation process followed the kinetics of second order reaction (Fig. 2). Calculated kinetic parameters for the oxidation reaction for individual fluoroquinolones suggest that the oxidation process is dependent on the pH of the reaction medium and the type of fluoroquinolone tested (Table 2). In solutions at pH = 4.5 PEF oxidizes quickly – $k = 10.00 \times 10^{-4}$/min, DAN and ENR – $k = 2.00 \times 10^{-4}$/min in similar way but slower than MAR – $k = 3.00 \times 10^{-4}$/min, while the slowest oxidation was observed for ORB – $k = 0.25 \times 10^{-4}$/min. The oxidation processes described by values $t_{0.1}$ and $t_{0.5}$ determining the time after which the concentration of the tested fluoroquinolone decreases, show that oxidation will proceed in the following order PEF > MAR > DAN =

Table 7. Products of oxidation of ORB induced by $KMnO_4$ in acidic conditions.

| Product | RT [min] | $[M + H]^+$ | Fragmentation ions | Proposed structure |
|---|---|---|---|---|
| **OP-1** | 4.65 | 400.1 | 356.1, 328.1, 310.1, 292.1, 268.1, 241.1, 227.1 |  |
| **OP-2** | 6.20 | 410.1 | 392.1, 382.1, 366.1, 364.1, 338.2, 298.1, 227.1 |  |
| **OP-3** | 6.90 | 412.2 | 394.1, 350.2, 334.1, 308.1, 267.1 |  |
| **ORB** | 7.44 | 344.1 | 378.1, 352.2, 336.1, 335.1, 321.1, 307.1, 295.1, 267.1, 255.1, 227.1 |  |

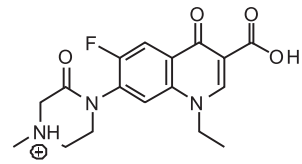
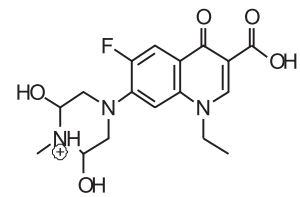
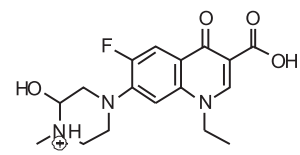
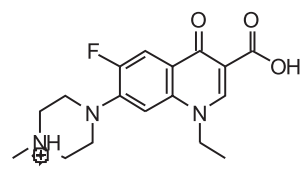
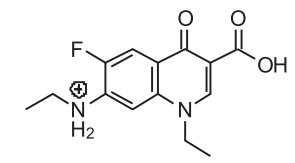
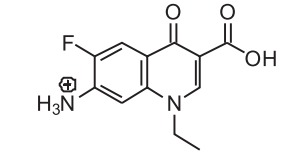
ENR > ORB (Table 3). Results of these studies confirm the degradation profiles, described by the equation ln k = f (pH ), which are different and dependent on the pH of the solutions of the individual compounds and the type of examined fluoroquinolone from the smallest ORB to the largest PEF (Fig. 3).

**Identification of oxidation products**

The identification of oxidation products of five fluoroquinolones (DAN, ENR, MAR, ORB and PEF) induced by $KMnO_4$ in acidic conditions was performed on a basis of UPLC/MS analysis and supported by fragmentation patterns obtained from MS/MS experiments. As we have reported in our earlier paper (7), the oxidation process mainly affect the 7-amine substituent of fluoroquinolone moiety, i.e., 5-ethyl-2,5-diazabicyclo[2.2.1]heptane, 4-ethylpiperazine, 4-methylpiperazine and 3,5-dimethylpiperazine, while the fluoroquinolone core remains unchanged.

It was found that the main route of oxidation involved hydroxylation in the close vicinity of *N*-1 and

Table 8. Products of oxidation of PEF induced by $KMnO_4$ in acidic conditions.

| Product | RT [min] | $[M + H]^+$ | Fragmentation ions | Proposed structure |
|---|---|---|---|---|
| **PP-1** | 3.41 | 348.1 | 330.1, 302.1, 276.2, 245.1, 233.1 |  |
| **PP-2** | 4.03 | 366.2 | 348.1, 330.1, 312.1, 286.1, 260.1, 232.1, 218.1 |  |
| **PP-3** | 4.54 | 350.2 | 332.1, 288.2, 277.1, 260.1, 245.1, 231.1, 217.1, 207.1 |  |
| **PEF** | 5.26 | 334.2 | 316.2, 290.2, 233.1, 205.1 |  |
| **PP-4** | 7.70 | 279.1 | 261.1, 235.1, 207.1, 179.1, 151.1 |  |
| **PP-5** | 7.97 | 251.1 | 233.1, 207.1, 179.1 |  |

*N*-4 piperazine atoms to respective hydroxylated derivatives and subsequent oxidation to their oxo-counterparts. For ENR, MAR and PEF, the main product of oxidation was 3-hydroxy derivative (EP-4, MP-3, PP-3), and 3,5-dihydroxy derivatives were also observed (EP-3, MP-2, PP-2). In case of ENR and MAR, the hydroxylactam derivatives (EP-2, MP-7), as products of further oxidation of piperazine moiety, were observed, although for ENR the intermediate step was dealkylation of *N*-4 nitrogen atom. For DAN the most abundant product of oxidation was *N*-4 demethylated 2,3,5-trihydroxy derivative (DP-2), mono-hydroxy or di-hydroxy derivatives were not observed. In case of ORB, possessing piperazine moiety with 3 and 5 position substituted with methyl group, the main product of oxidation was 2-hydroxy derivative (OP-3). Oxidation of piperazine moiety of ORB in close vicinity to more basic *N*-4 nitrogen atom, leading to 3,5-dihydroxy derivative (OP-1), involved demethylation by oxidative C-C bond cleavage, and was 10-fold less effective than oxidation in position 2. Oxidative C-C bond cleavage occurred also in case of DAN, leading to 1,2,3,4-tetrahydropyrazine derivative (DP-3). Further oxidation of fluoroquinolones led to ring opening, dealkylation (e.g., MP-4, MP-5, MP-6, PP-4) and finally yielded 7-amino quinolone products (DP-4, EP-5, PP-5). This product was not observed in case of MAR and OFL, probably due to the presence of electron-withdrawing substituents in both *ortho* positions of phenyl ring, i.e., fluorine and oxygen atoms in case of MAR and two fluorine atoms in case of OFL, and hence lower basicity of *N*-1 nitrogen atom of piperazine moiety than in case of the other investigated fluoroquinolones.

The structures of presented stable oxidation products were confirmed by Collisionally Activated Decomposition (CAD) experiments. The fragmentation pattern involved loss of $H_2O$ from the carboxylate group of quinolone, then loss of carboxylate function and piperazine ring degradation. Quinolone *N*-1 nitrogen dealkylation proceeded slowly, except for MAR, for which 3,4-dihydro-2*H*-1,3,4-oxadiazine ring easily underwent degradation processes. For ORB, cyclopropyl moiety seems to be stable, what may be attributed to an influence of electron-withdrawing of additional fluorine substituent in position 8 of quinolone. Proposed structures of oxidation products of the examined fluoroquinolones are presented in Tables 4-8, for DAN, ENR, MAR, ORB, and PEF, respectively.

## CONCLUSIONS

The elaborated method complies with the acceptance criteria for methods which can be useful for the determination of DAN, ENR, MAR, ORB and PEF in the presence of its oxidation products. The method was completely validated showing satisfactory data for all the parameters tested. Executed studies have shown that an oxidation process followed kinetics of the second order reaction for the substrates. Kinetic parameters such as rate constants $k$ and the times $t_{0.1}$ and $t_{0.5}$ depended on solution acidity and type of studied fluoroquinolones.

It was found, that the most susceptible fluoroquinolones for oxidation was PEF. DAN, ENR and MAR displayed lower degradation rate with comparable $t_{0.1}$ and $t_{0.5}$ values, while ORB was the most stable among the tested drugs. The proposed model of investigation of oxidation profile for presented fluoroquinolones may be regarded as a predictor of their stability.

Degradation of fluoroquinolones mainly affected piperazine moiety giving respective hydroxy- and oxo-derivatives. Further oxidation of DAN, ENR, MAR and PEF, following dealkylation, and degradation of piperazine yielded 7-amino fluroquionolones analogs.

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# DEVELOPMENT OF A QUALITY CONTROL METHOD FOR SCHISANDRAE CHINENSIS FRUCTUS WITH MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

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**Abstract**: The ripened fruits of *Schisandra chinensis* (Turcz.) Baill., called Schisandrae Chinensis Fructus, are commonly used in traditional herbal medicine with a long history. The lignan constituents, especially the dibenzocyclooctadiene-type lignans, are mostly responsible for the biological and pharmacological effects of this herbal medicine. In this paper, a new micellar electrokinetic capillary chromatography (MECC) method to analyze the active components of lignans is reported. The separating conditions for analysis of the lignans were optimized using a buffer solution consisting of 70 mM sodium dodecyl sulfate (SDS), 35% acetonitrile (v/v), and 100 mM Tris (pH 9.50). The applied voltage was 27 kV (positive polarity), and the capillary temperature was 25°C. Among the common peaks in the HPCE fingerprint, six peaks were identified as schizandrin, schizandrol B, schisantherin, deoxyschisandrin, γ-schisandrin and schizandrin C. Based on HPCE fingerprint and content determination, the quality of fifteen samples from different areas of China was objectively assessed. In summary, a new HPCE-based method was developed for the quality control of Schisandrae Chinensis Fructus, which could be very helpful for the proper use of Schisandrae Chinensis Fructus.

**Keywords:** Schisandrae Chinensis Fructus, micellar electrokinetic capillary chromatography, fingerprint, quality control

The ripened fruit of Schisandrae Chinensis Fructus has unique properties and has been widely used in China, Korea, Japan and other Asian countries to treat diseases and improve health. As reported previously, the ripened fruits of Schisandrae Chinensis Fructus mainly contain three classes of chemical constituents, namely lignans (1, 2),organic acid, and volatile oils. Schisandra lignans are the major constituents of Schisandrae Chinensis Fructus. More than forty have been isolated, among the most abundant are: schizandrin, schizandrol B, schisantherin, deoxyschisandrin, γ-schisandrin and schizandrin C. Lignans are largely responsible for the beneficial effect of schisandra extracts (3), which can prevent hepatic toxicity and virus infection, reduce oxygen radical generation, inhibit inflammation and so on (4-7).

So far, many approaches have recently been developed for the qualitative and quantitative analysis of the major lignans in Schisandrae Chinensis Fructus. Among these, the reversed-phase high performance liquid chromatography (RP-HPLC) with UV detection and thin-layer chromatography methods are most frequently used (8). Nevertheless, those methods are time consuming and require excessive solvent.

In past years, great advance has been made in capillary electrophoresis (CE). Due to rapid analytical speed, high resolution and low-loss of solvent, this method has been widely used in the analysis of proteins and peptides (9-12). Presently, this analytical method has a tendency of being used in the quality assessment of drugs, especially for traditional herbs, because there are many uncharged ingredients in traditional herbs and the uncharged molecules can be analyzed by micellar electrokinetic capillary chromatography (MECC). Unfortunately, the quality control of Schisandrae Chinensis Fructus by CE has not been well investigated yet.

To address these deficiencies, a high separation-capable MECC method was generated for the separation of lignans in this study. Further, in order

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to comprehensively evaluate the quality of Schisandrae Chinensis Fructus, fingerprints were established based on the chromatography from 15 sample batches collected from northern China, and the contents of six lignans in the herb were simultaneously determined by MECC method as well.

## EXPERIMENTAL

### Chemical and reagents

The major lignans including schizandrin, schizandrol B, schisantherin, deoxyschisandrin, γ-schisandrin and schizandrin C (Fig. 1) were isolated from Schisandrae Chinensis Fructus as described elsewhere (13, 14). The chemical structures of these compounds were characterized by several methods, including UV, IR, NMR and MS. The purity of the compounds, as determined by HPLC, was > 98%. Analysis-grade sodium dodecyl sulfate (SDS) and tris(hydroxymethyl)aminomethane (TRIS) were purchased from Sigma company (St. Louis, MO, USA), HPLC-grade acetonitrile was purchased from Tedia (Fairfield, USA) and high-purity deionized water (18.2 MΩ cm) was obtained from a Millipore system (Millipore, Bedford, MA, USA). Analysis-grade methanol used for sample extraction, sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Shanghai Chemical Corporation of China.

### Plant material

The samples (S1 - S15) of Schisandrae Chinensis Fructus were collected from Heilongjiang, Liaoning and Jilin province of China during early autumn and identified by professor Lu Tuling of Nanjing University of Chinese Medicine. Voucher specimens were deposited at College of Pharmacy of Nanjing University of Chinese Medicine. Details of the samples are listed in Table 1.

### CE equipment and conditions

MECC analysis was performed with Agilent series G1600 AX equipment consisting of automatic injector, temperature controller, and diode array detector (DAD). Data analysis was performed by means of the CE ChemStation software. Fused-silica capillaries (Agilent Company, USA) of 75 μm ID, 64.5 cm total length and 56.0 cm effective length were used for MECC analysis. Injection was accomplished by the application of 50 mbar for 4 s. The background electrolyte solution was prepared by 40 mM SDS and 35% acetonitrile in 100 mM Tris buffer, then, the pH of the solution was adjusted to 9.5 with 0.2 M HCl to make up the running electrolyte. Separation were performed at 27 kV ( positive polarity ) and 25°C column temperature. Samples were detected by DAD at 250 nm. To ensure good reproducibility, the capillaries were washed by 0.1

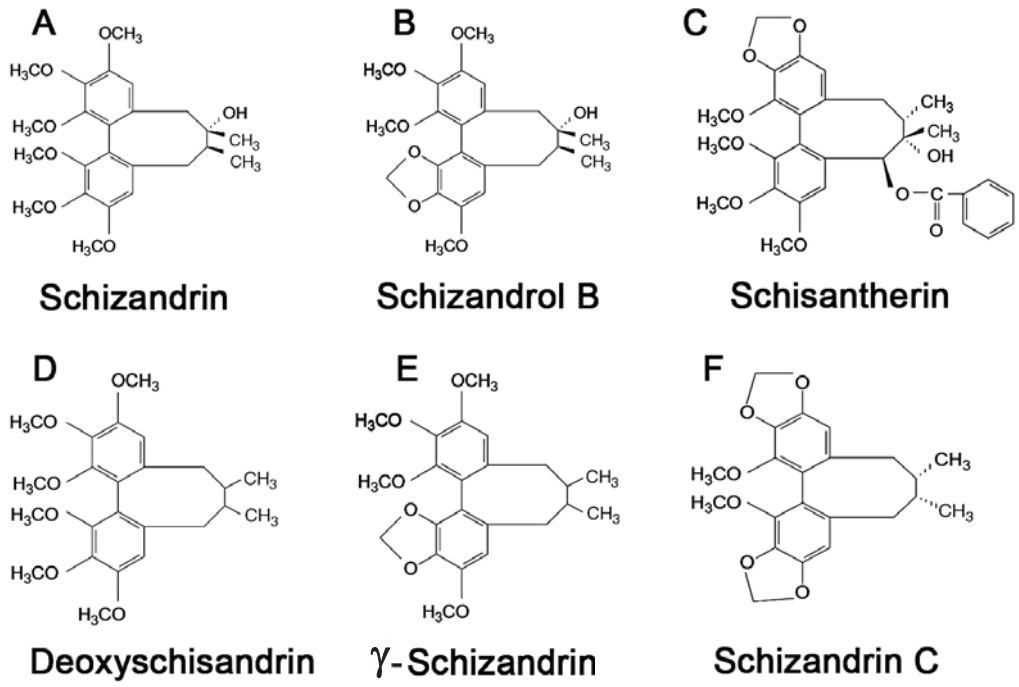


Figure. 1. Chemical structures of the standard compounds in Schisandrae Chinensis Fructus

Table 1. Details of the herbal materials collected.

| No. | Producing area | Species | Collection data |
|---|---|---|---|
| 1 | Xinglin, Heilongjiang | *Fructus Schisandrae Chinensis* | 23. 10. 2012 |
| 2 | Qiqihaer, Heilongjiang | *Fructus Schisandrae Chinensis* | 01. 11. 2012 |
| 3 | Yichun, Heilongjiang | *Fructus Schisandrae Chinensis* | 11. 11. 2012 |
| 4 | Xinganlin, Heilongjiang | *Fructus Schisandrae Chinensis* | 13. 10. 2013 |
| 5 | Mudanjiang, Heilongjiang | *Fructus schisandrae Chinensis* | 26. 10. 2013 |
| 6 | Teli, Heilongjiang | *Fructus Schisandrae Chinensis* | 05. 11. 2013 |
| 7 | Mishan, Heilongjiang | *Fructus schisandrae Chinensis* | 09. 11. 2013 |
| 8 | Qingan, Heilongjiang | *Fructus Schisandrae Chinensis* | 16. 11. 2013 |
| 9 | Zhaoqing, Heilongjiang | *Fructus Schisandrae Chinensis* | 23. 11. 2013 |
| 10 | Jian, Niaoning | *Fructus Schisandrae Chinensis* | 25. 11. 2013 |
| 11 | Kuandian, Niaoning | *Fructus Schisandrae Chinensis* | 26. 11. 2012 |
| 12 | Wusong, Changbaishan, Jilin | *Fructus Schisandrae Chinensis* | 05. 11. 2012 |
| 13 | Chibei, Changbaishan, Jilin | *Fructus Schisandrae Chinensis* | 06. 11. 2013 |
| 14 | Songjianghe, Jilin | *Fructus Schisandrae Chinensis* | 23. 11. 2013 |
| 15 | Wangqin, Jilin | *Fructus Schisandrae Chinensis* | 28. 11. 2013 |

M NaOH for 3 min, deionized water for 5 min, then running electrolyte for 5 min.

**Standard preparation**

Stock solutions of each lignan were prepared by dissolving the precisely weighed standards into 10 mL methanol, then, portions of the six solutions were combined, evaporated to dryness, and the residue was dissolved in 2 mL background buffer solution (40 mM SDS and 35% acetonitrile in 100 mM Tris buffer). Concentrations of the six compounds in the background buffer solution were 113 μg/mL for schizandrin, 87.5 μg/mL for schizandrol B, 93.75 μg/mL for schisantherin, 85.75 μg/mL for deoxyschisandrin, 95.0 μg/mL for γ-schisandrin and 57 μg/mL for schizandrin C.

**Sample preparation**

The dried Schisandrae Chinensis Fructus were pulverized and sieved (60 mesh). Samples (0.2 g) were extracted with methanol (50 mL) for 30 min by ultrasonication (250 W, 30 kHz) and the extraction was repeated twice. Extracts were combined, filtered, evaporated to dryness, and the residue was dissolved in 2 mL of background buffer solution (40 mM SDS and 35% acetonitrile in 100 mM Tris buffer). Solutions were filtered through a 0.22 μm polytetrafluoroethylene membrane filter before analysis.

**Data analysis**

Similarity analysis was performed by use of the professional software "Similarity Evaluation System (SES) for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2004A)" recommended by Chinese Pharmacopoeia Commission. This software is currently not commercially available, but the Chinese Pharmacopoeia Commission allows free use of this software, solely for quality control of Chinese herbal medicine. The principle of the software is to evaluate the similarity of different chromatograms by calculating the correlation coefficient and/or multivariate cosine.

**RP-HPLC analysis**

HPLC analysis was performed with Agilent series 1100 equipment consisting of G1311A quaternary pump, G1315B-DAD, and G1316A column compartment. Samples were separated on a Kromasil (Sweden) $C_{18}$ column (4.6 mm i.d. × 250 mm, 5 μm particle). The mobile phase was a gradient prepared from $H_2O$ (component A) and $CH_3CN$ (component B), and the conditions used for gradient elution were: 0–15 min, 2–55% $CH_3CN$; 15–30 min, 55–60% $CH_3CN$; 30–50 min, 60–70% $CH_3CN$; 50–60 min, 70–100% $CH_3CN$; 60–70 min, 100% $CH_3CN$. The flow rate was 1.0 mL/ min. After the 100% acetonitrile, the mobile phase was switched to 2% acetonitrile for 10 min to equilibrate the column before the next sample was injected. The injection volume was 10 μL. Chromatograms with good separation were obtained when the column temperature was 30°C. The detection wavelength was 250 nm (15).

## RESULTS AND DISCUSSION

### Optimization of the extraction conditions

To achieve maximum recovery of the components in Schisandrae Chinensis Fructus, the extraction conditions, including solvent and method, were optimized. First, petroleum ether, n-hexane, ethanol, methanol, 95% ethanol, 75% ethanol, 95% methanol and 75% methanol were evaluated as extracted solvents. Methanol was shown to be the best solvent, because it enabled maximum extraction of the most active components with relatively high yield. To examine the effect of extraction method on recovery, ultrasonic extraction, Soxhlet extraction and extraction under reflux were investigated. Although similar recovery was achieved by use of all three methods, ultrasonic extraction was selected mainly because of its relatively simple handling procedure. The samples were eventually extracted twice with 30 min for each time.

Notably, the capillaries were usually blocked if the sample extraction solution was directly injected. This problem can be circumvented by evaporating the extracts to dryness and dissolving the residue in 2 mL of background buffer solution.

### Optimization of the MECC separation conditions

The β-CD is a frequently used substance in MECC separation, because its special structure allows the formation of inclusion compounds that consists of β-CD and the analytes (16, 17). In the experiments, when the β-CD of different concentrations (5, 10, 15, 20, 25, 30 mmol/L) were added to tetraborate systems, the separation of the main lignans did not improve noticeably, and meanwhile, many air bubbles were produced in the β-CD system. Therefore, β-CD is not suitable for the analysis.

Besides β-CD, SDS surfactants are also widely used in MECC (18, 19). Both SDS-tetraborate and SDS-Tris systems were used in analyses. While SDS-tetraborate was advantageous in the analysis of small molecular organic compounds, SDS-Tris was preferentially used in protein analysis. The results shown that the SDS-Tris system was better employed in analyzing the lignans of Schisandrae Chinensis Fructus by MECC, as compared with the SDS-tetraborate system, because a good separation of the lignans was achieved under SDS-Tris system.

In order to optimize SDS concentration, effect of SDS at concentrations ranging from 10 to 80 mmol/L on lignan separation was investigated. The results demonstrated that the lignans could not be nicely separated in the presence of < 30 mmol/L SDS. However, if SDS was used at high concentrations, Joule heat was high, and further, more bubbles were produced. In addition, SDS at high concentrations led to prolonged migration time for the analytes and peak broadening. As such, the optimal concentration for SDS in the analysis of lignans was finally set at 40 mmol/L.

The influence of TRIS concentration on lignan separation was further explored. In this experiment, TRIS concentration varied from 10 to 150 mmol/L. The results demonstrated that the resolution of peaks was increasingly improved as the relative concentration of TRIS in the buffer system increased, and the optimal concentration for TRIS was finally set at 100 mmol/L.

Because of the lipophilicity of the lignans, suitable amounts of organic modifiers which can promote the dissolution of the lipophilic compounds were added to the background buffer. In this study, methanol and acetonitrile were used as organic modifiers and their effects on peaks resolution were examined. The results demonstrated that the migration behavior of acetonitrile was better than that of methanol. The effect of acetonitrile at different concentrations was further examined. The result revealed that 35% of acetonitrile was found to provide the best separation.

Table 2. Linearity calibration curves of the determined components.

| No. | Identification | Regression equation | R (n = 6) | Linear ranges (μg/mL) | LOD (μg/mL) | LOQ (μg/mL) |
|---|---|---|---|---|---|---|
| 1 | Schizandrin | y = 6.982x + 28.994 | 0.9992 | 11.3-45.20 | 0.45 | 0.13 |
| 2 | Schizandrol B | y = 5.733 x + 19.112 | 0.9992 | 8.75-35.00 | 0.44 | 0.13 |
| 3 | Schisantherin | y = 3.655 x + 11.221 | 0.9995 | 4.69-18.75 | 0.47 | 0.14 |
| 4 | Deoxyschisandrin | y = 4.224 x + 18.702 | 0.9995 | 8.58-34.30 | 0.43 | 0.13 |
| 5 | γ-Schisandrin | y = 6.229 x + 10.002 | 0.9991 | 9.50-38.00 | 0.48 | 0.14 |
| 6 | Schizandrin C | y = 5.429 x + 18.868 | 0.9992 | 2.85-11.40 | 0.29 | 0.09 |

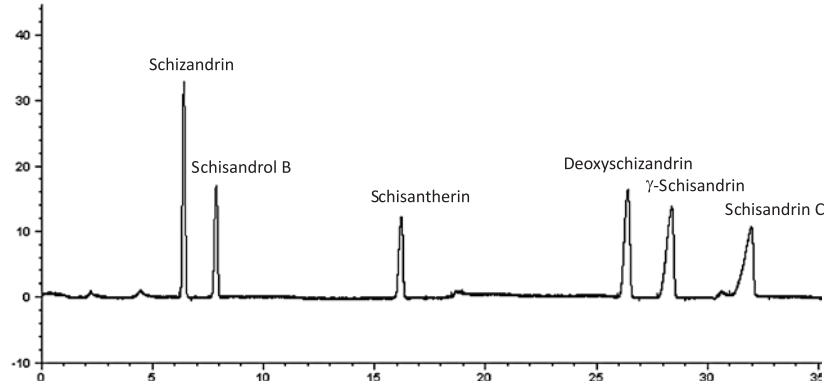


Figure 2. Chromatograms obtained from the standard compounds. The compounds corresponding to peaks are schizandrin, schisandrol B, schisantherin, deoxyschizandrin, γ-schisandrin and schisandrin C, respectively

Table 3a. Precision, repeatability and recovery data of six lignan compounds by the proposed HPCE method.

| Compound | Precision | | | | Reproducibility | | Stability | | Accuracy | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Intra-day | | Inter-day | | | | | | | |
| | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) | Mean (%) | RSD (%) |
| Schizandrin | 0.422 | 1.666 | 0.423 | 2.723 | 0.420 | 3.646 | 0.419 | 2.646 | 101.32 | 3.22 |
| Schizandrol B | 0.083 | 1.822 | 0.082 | 2.833 | 0.082 | 3.994 | 0.081 | 2.867 | 98.23 | 3.76 |
| Schisantherin | 0.024 | 2.036 | 0.023 | 1.996 | 0.023 | 3.877 | 0.023 | 3.456 | 97.54 | 3.08 |
| Deoxyschisandrin | 0.094 | 2.432 | 0.094 | 2.655 | 0.095 | 3.664 | 0.094 | 3.273 | 101.32 | 3.01 |
| γ-Schisandrin | 0.212 | 2.177 | 0.212 | 2.277 | 0.213 | 2.245 | 0.213 | 3.163 | 101.37 | 3.54 |
| Schizandrin C | 0.017 | 2.321 | 0.018 | 2.435 | 0.018 | 3.082 | 0.018 | 3.002 | 102.33 | 3.45 |

Mean (%): the average percentage content of the compound. Recovery (%) = (amount found – original amount )/ amount spiked × 100%

Table 3b. Precision, repeatability and stability data of fingerprint by the proposed HPCE method.

| Similarity value | Precision | | | | Reproducibility | | Stability | |
|---|---|---|---|---|---|---|---|---|
| | Intra-day | | Inter-day | | | | | |
| | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) | Mean | RSD (%) |
| | 0.999 | 0.687 | 0.999 | 0.929 | 0.996 | 1.203 | 0.998 | 1.106 |

Mean: the average similar value of the samples.

The pH value is also an important parameter for lignans separation. In this study, the effect of pH value ranging from 9.0 to 10.0 was examined. The results showed that the peaks resolution was getting better as the pH values increased from 9.1 to 9.5. However, when the pH value was above 9.5, the resolution decreased, probably because high pH value led to heat overproduction. Therefore, the optimal pH value was 9.5.

The effects of voltages ranging from 18 kV to 28 kV on peaks resolution in MECC analysis were also examined. The results revealed that the optimal voltage was 27 kV because it gave the best peak resolution and the shortest analytical time.

**Calibration and validation**

Six concentrations of the standard solutions were analyzed in triplicate, and calibration curves were constructed from peak areas of standards *versus* their concentrations. The standard solutions were diluted to a series of appropriate concentrations with acetonitrile. The limits of determinations

(LODs) (S/N ≈ 3) and limits of quantifications (LOQs) (S/N ≈ 10) under the present conditions were determined. The calibration data, linear ranges, R, LOD, and LOQ values are listed in Table 2. The data showed that there was a good relationship between the concentrations of the six compounds measured and their peak areas within the test range (R > 0.999).

The optimized conditions were subjected to method validation. The intra-day and inter-day precisions of the content determination method were evaluated by analysis of the standard solution during a single day or three consecutive days, respectively. The reproducibility of the content determination method was evaluated by analysis of six replicates of the same sample. The same sample solution was also analyzed nine times consecutively at different times after preparation (0, 4, 8, 12, 16, 20, 24, 28, and 32 h) to test its stability. Accuracy of content determination was determined in recovery experiments in which the standard compounds of 80, 100 and 120% of the sample content were added to approximately 0.1 g of sample (S1) and then extracted and analyzed. Data analysis of the chromatograms was performed for six main lignan components as shown in Table 3a. Data analysis of the chromatographic fingerprints was performed by use of SES software, and similarities between chromatograms were used to evaluate fingerprint quality, which determines the total quality of the drug. The results from this analysis are listed in Table 3b. According to the result of the test, the analytical method was effective for simultaneous determination of the six compounds with fingerprint evaluation in Schisandrae Chinensis Fructus.

## RESULTS

Under the optimized MECC conditions, a mixture of the standard lignans, including schizandrin, schizandrol B, schisantherin, deoxyschisandrin, γ-schisandrin, and schizandrin C were separated, as shown in Figure 2. Further, fifteen batches of Schisandrae Chinensis Fructus samples collected from different producing areas in China were also analyzed by MECC.

### Determination of lignan content in the herbs

The contents of six compounds of the fifteen batch samples were determined using optimized MECC conditions, and are listed in Table 4. Chromatograms obtained from fifteen samples from different producing areas were overlaid in Figure 3. In these analytes, schizandrin was the most abun-

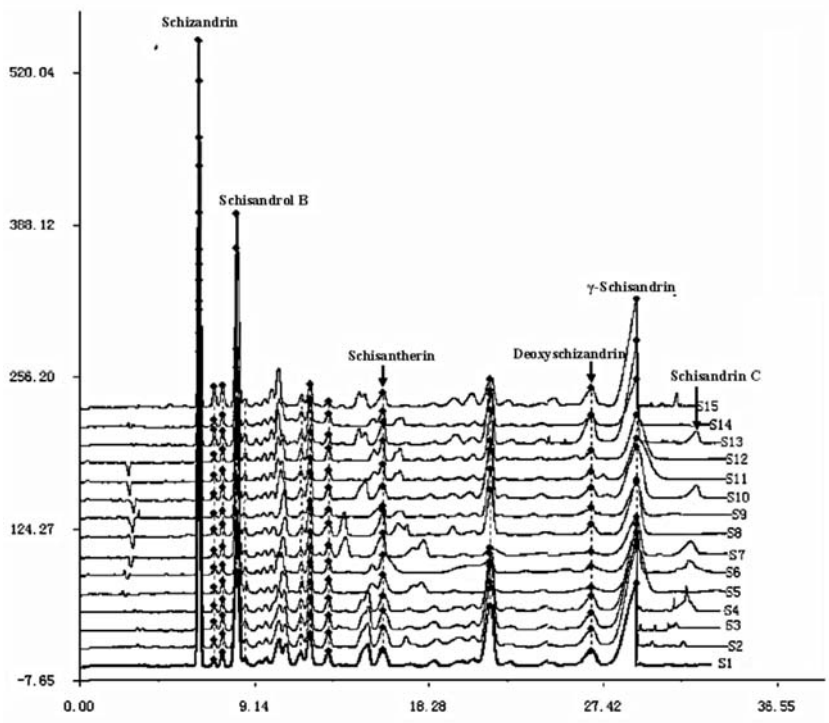


Figure 3. Overlaid chromatograms obtained from 15 samples from different locations. Samples 1-9 were from Heilongjiang province, Samples 10, 11 were from Niaoning province, samples 12-15 were from Jilin province

Table 4. The content of six main lignnans compounds from 15 samples (n = 3).

| No. | The percentage content of six main lignans compounds (%) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Schizandrin | Schizandrol B | Schisantherin | Deoxyschisandrin | γ-Schisandrin | Schizandrin C | Total lignans |
| S1 | 0.513 ± 0.005 | 0.101 ± 0.002 | 0.034 ± 0.0010 | 0.109 ± 0.002 | 0.325 ± 0.002 | ND | 1.082 ± 0.02 |
| S2 | 0.403 ± 0.004 | 0.099 ± 0.002 | 0.034 ± 0.0009 | 0.198 ± 0.002 | 0.301 ± 0.002 | 0.028 ± 0.0008 | 1.064 ± 0.02 |
| S3 | 0.477 ± 0.006 | 0.111 ± 0.001 | 0.024 ± 0.0005 | 0.149 ± 0.001 | 0.214 ± 0.003 | ND | 0.975 ± 0.01 |
| S4 | 0.415 ± 0.006 | 0.089 ± 0.001 | 0.032 ± 0.0008 | 0.192 ± 0.002 | 0.118 ± 0.002 | ND | 0.847 ± 0.01 |
| S5 | 0.545 ± 0.004 | 0.123 ± 0.003 | 0.030 ± 0.0008 | 0.094 ± 0.001 | 0.226 ± 0.001 | ND | 1.018 ± 0.02 |
| S6 | 0.412 ± 0.005 | 0.080 ± 0.002 | 0.019 ± 0.0004 | 0.135 ± 0.002 | 0.338 ± 0.002 | 0.023 ± 0.0006 | 1.007 ± 0.01 |
| S7 | 0.428 ± 0.004 | 0.119 ± 0.002 | 0.024 ± 0.0005 | 0.094 ± 0.001 | 0.214 ± 0.002 | 0.013 ± 0.0003 | 0.892 ± 0.01 |
| S8 | 0.578 ± 0.006 | 0.211 ± 0.005 | 0.013 ± 0.0003 | 0.198 ± 0.002 | 0.308 ± 0.002 | ND | 1.308 ± 0.02 |
| S9 | 0.534 ± 0.007 | 0.208 ± 0.004 | 0.016 ± 0.0003 | 0.138 ± 0.001 | 0.324±0.003 | ND | 1.220 ± 0.02 |
| S10 | 0.468 ± 0.005 | 0.123 ± 0.003 | 0.034 ± 0.0008 | 0.109 ± 0.002 | 0.198 ± 0.002 | 0.024 ± 0.0005 | 0.956 ± 0.01 |
| S11 | 0.459 ± 0.004 | 0.134 ± 0.001 | 0.024 ± 0.0009 | 0.108 ± 0.002 | 0.255 ± 0.003 | ND | 0.980 ± 0.02 |
| S12 | 0.498 ± 0.003 | 0.102 ± 0.002 | 0.046 ± 0.0011 | 0.219 ± 0.001 | 0.286 ± 0.002 | ND | 1.151 ± 0.02 |
| S13 | 0.474 ± 0.003 | 0.168 ± 0.002 | 0.043 ± 0.0014 | 0.123 ± 0.001 | 0.389 ± 0.003 | 0.019 ± 0.0004 | 1.216 ± 0.01 |
| S14 | 0.458 ± 0.006 | 0.099 ± 0.001 | 0.020 ± 0.0004 | 0.099 ± 0.001 | 0.351 ± 0.002 | ND | 1.027 ± 0.02 |
| S15 | 0.518 ± 0.004 | 0.145 ± 0.001 | 0.024 ± 0.0003 | 0.103 ± 0.002 | 0.228 ± 0.002 | ND | 1.019 ± 0.02 |

Total lignans: the sum of 6 average contents of lignans (Schizandrin, Schizandrol B, Schisantherin, Deoxyschisandrin, γ-Schisandrin and Schizandrin C). ND: not detected.

Table 5. Similarity values of 15 samples from different areas.

| No. | Similarity value | No. | Similarity value |
|---|---|---|---|
| S1 | 0.928 | S9 | 0.918 |
| S2 | 0.919 | S10 | 0.901 |
| S3 | 0.902 | S11 | 0.943 |
| S4 | 0.912 | S12 | 0.952 |
| S5 | 0.992 | S13 | 0.927 |
| S6 | 0.994 | S14 | 0.943 |
| S7 | 0.936 | S15 | 0.942 |
| S8 | 0.924 | | |

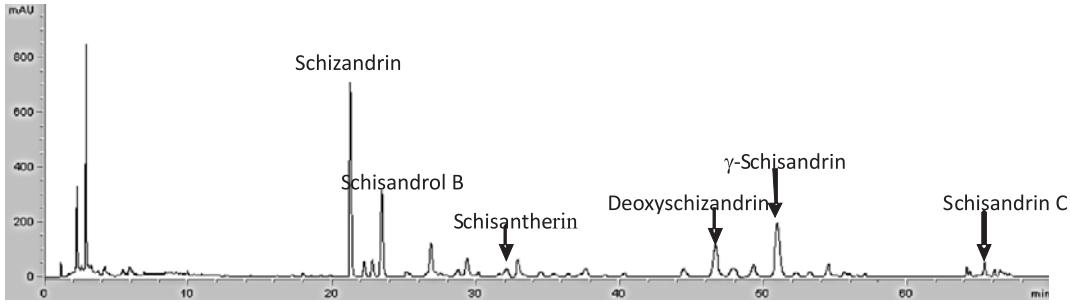


Figure 4. HPLC chromatogram of the extraction with optimized conditions. Samples were separated on a Kromasil (Sweden) $C_{18}$ column (4.6 mm i.d. × 250 mm, 5 μm particles). The mobile phase was a gradient prepared from $H_2O$ (component A) and $CH_3CN$ (component B) and the conditions used for gradient elution were: 0–15 min, 2–55% $CH_3CN$; 15–30 min, 55–60% $CH_3CN$; 30–50 min, 60–70% $CH_3CN$; 50–60 min, 70–100% $CH_3CN$; 60–70 min, 100% $CH_3CN$. The flow rate was 1.0 mL/min. After the 100% acetonitrile, the mobile phase was switched to 2% acetonitrile for 10 min to equilibrate the column, then the next sample was injected. The injection volume was 10 μL. Chromatograms with good separation were obtained when the column temperature was 30°C. The detection wavelength was 250 nm

dant constituent in all of the samples, with a content that varied from 0.403 to 0.518% and occupied 37.9 to 53.5% of the total lignan contents. The γ-schisandrin ranked as the second main constituent with content that varied from 0.118 to 0.389%, followed by deoxyschisandrin, schizandrol B, and schisantherin. Schizandrin C was the compound with the least content, which could not be detected in ten samples including six batches from Heihongjiang (S1, S3, S4, S5, S8, S9), one batch from Liaoning (S11), and three batches from Jilin (S12, S14, S15).

**Fingerprints of the lignans in the plants**

In the study, the common pattern of HPCE fingerprints was established by using SES software. More than twenty peaks were separated in all sample batches, but only twelve peaks could be regarded as common peaks, of which RSD of the peaks' relative retention times for all batches was less than 1%, and the peaks belonged to the same substance. The overlaid chromatographs, as shown in Figure 3, provided a general profile of the compounds in these samples.

Among these peaks, schizandrin, schizandrol B, schisantherin, deoxyschisandrin, γ-schisandrin, and schizandrin C were identified by comparing the migration times and UV spectra of peaks with the six standards, respectively. Furthermore, spiking samples with the standards further supported the identification of the lignan peak. Peak of schizandrin C did not belong to the common peaks, because it could not be detected in ten batches of samples. As a result, the similarity value for the fifteen batches of samples was above 0.99 (Table 5).

**RP-HPLC analysis**

The sample extract could be separated by HPLC under the optimized HPLC conditions, as

listed in section 2.7, but the analytical time for sample was 70 min, which was longer than the one from HPCE analysis. The chromatogram of sample 2 from Heilongjiang obtained with the optimized conditions is shown in Figure 4. As compared with RP-HPLC, HPCE shortened the analytical time and reduced the reagents consumption, which is more advantageous for herb ingredient analysis.

## CONCLUSION

In this work, a MECC-based method was developed for the analysis of lignans from Schisandrae Chinensis Fructus. As compared with HPLC, this proposed method is simple and has characteristics of high separation efficiency, rapid analytical time, and low cost. With the optimal separation conditions, fingerprint and the content determination were successfully performed for evaluating the quality of Schisandrae Chinensis Fructus. Thus, this method can be adopted for the quality control of lignans of Schisandrae Chinensis Fructus.

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# DETERMINATION OF LIPOPHILICITY PARAMETERS OF NEW DERIVATIVES OF $N^3$-SUBSTITUTED AMIDRAZONES BY REVERSED PHASE THIN LAYER CHROMATOGRAPHY

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**Abstract:** The retention behavior of 23 derivatives of $N^3$-substituted amidrazones with potential pharmacological activity were evaluated by reverse-phase thin layer chromatography. Examined compounds were divided into three groups depending on molecular structure and analyzed using methanol/water and methanol/water/acetic acid mobile phases on RP18 HPTLC plates. The linear relationship between retention parameter $R_M$ and the percentage composition of methanol has been obtained within all groups in whole examined concentration range, which permitted determination of lipophilicity parameters: $R_{M0}$ by extrapolation and $\varphi_0$ by interpolation. $R_{M0}$ values obtained within groups were compared with log P values obtained by eight computational algorithms (KOWWIN, XLOGP2, XLOGP3, ALOGPS, MLOGP, ALOGP, AC logP, miLogP).

**Keywords:** amidrazones, triazoles, thin-layer chromatography, lipophilicity

Amidrazones – hydrazones of acid amides – are useful precursors in the synthesis of many chemical compounds (1-3). In recent years, some derivatives of $N^3$-substituted amidrazones showed diverse biological activity: antiviral, antibacterial, immunomodulatory, anti-inflammatory, analgesic, antitumor and others (4-7).

Growing number of close-related amidrazone derivatives cause need to find a criteria which enable preliminary estimation of biological activity of obtained compounds and optimization of lead structures in the future. Lipophilicity is one of the crucial physicochemical properties that comprise influence of various molecular parameters of compounds and is highly correlated with biological effects of potential drugs (8, 9). It significantly affects both transport of compounds through membranes in a biological system and the formation of compound-receptor complex. Chromatographic methods using RP-18 phases are especially useful for lipophilicity analyses regarding similarity of stationary phase to biological membranes. RP-TLC is a rapid, easy to perform technique which requires small quantities of the sample and enables simultaneous analysis of several compounds. It is reliable method for bioavailability prediction of potential drugs, which provide useful informations to QSAR studies (10, 11).

In present work, we evaluated chromatographic lipophilicity parameters of 23 potentially active amidrazone derivatives using RP-TLC method and compared them with computed log P values.

## EXPERIMENTAL

Amidrazones derivatives **1-23** synthesized in Department of Organic Chemistry in Nicolaus Copernicus University in Bydgoszcz were initially divided into 3 groups (triazoles **1-10**, linear **11-16** and **17-23**) basing on their chemical structure (Table 1). Compounds were dissolved in methanol (3 mg/mL), samples (10 μL) of each class were applied on individual plates, then dried on air. Five mixtures of methanol and water (40, 45, 50, 55, 60% v/v) and the same mixtures with 0.2% acetic acid addition were used as mobile phases.

Chromatography was performed on HPTLC RP-18W nano-silica gel aluminium plates (60 Å medium pore diameter, $F_{254}$ aluminium sheets, Fluka, Germany, 0.150 mm thick layer). The plates (10 × 10 cm) were developed in horizontal DS-chamber (Chromdes, Lublin, Poland) using saturat-

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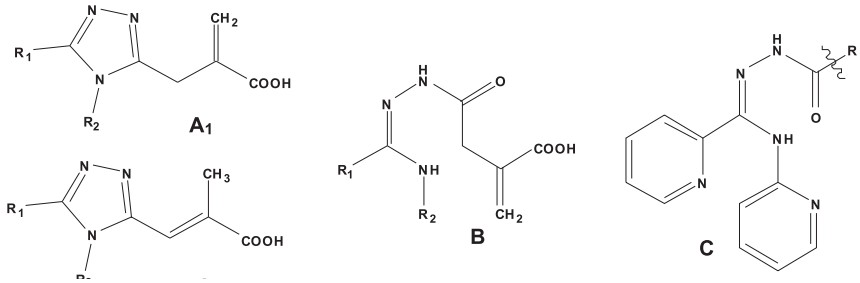
ed conditions (face-down, 30 min of saturation in ambient temperature). The developing distance was 8 cm. Developed plates were air dried and observed under 254 nm ultraviolet lamp. Compounds were localized by quenching the plate fluorescence. If multiple spots appeared, the plate was sprayed with 1% copper (II) sulfas solution in methanol/water 50/50% v/v mixture to visualize proper spots. All solvents and reagents were of analytical grade, and water freshly distilled. All experiments were performed in a room with stable temperature of 22°C.

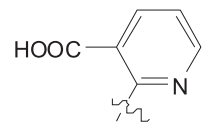
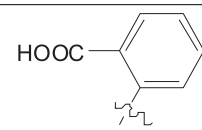
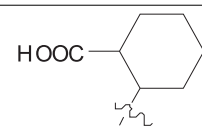
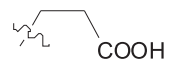
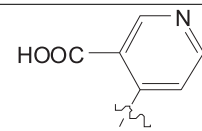
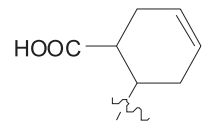
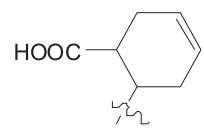
Regression and correlation analyses were performed by Statistica 10 software.

## RESULTS

In reverse-phased system, triazoles **1-10** showed single spots without the presence of impu-

Table 1. The structures of compounds **1-23**.




| Comp. | Core | $R_1$ | $R_2$ | Comp. | Core | R |
|---|---|---|---|---|---|---|
| **1** | $A_1$ | 2-$C_5H_4N$ | $C_6H_5$ | **17** | C |  |
| **2** | $A_2$ | 2-$C_5H_4N$ | $C_6H_5$ | | | |
| **3** | $A_1$ | 2-$C_5H_4N$ | 2-$C_5H_4N$ | **18** | C |  |
| **4** | $A_2$ | 2-$C_5H_4N$ | 2-$C_5H_4N$ | | | |
| **5** | $A_1$ | 2-$C_5H_4N$ | 4-$NO_2$-$C_6H_4$ | **19** | C |  |
| **6** | $A_1$ | 4-$C_5H_4N$ | 4-$CH_3$-$C_6H_4$ | | | |
| **7** | $A_1$ | $C_6H_5$ | $C_6H_5$ | **20** | C |  |
| **8** | $A_1$ | 4-$C_5H_4N$ | $C_6H_5$ | | | |
| **9** | $A_1$ | 2-$C_5H_4N$ | 4-$CH_3$-$C_6H_4$ | **21** | C |  |
| **10** | $A_1$ | $C_6H_5$ | 4-$NO_2$-$C_6H_4$ | | | |
| **11** | B | 2-$C_5H_4N$ | $C_6H_5$ | **22** | C |  |
| **12** | B | 2-$C_5H_4N$ | 2-$C_5H_4N$ | | | |
| **13** | B | 2-$C_5H_4N$ | 4-$NO_2$-$C_6H_4$ | | | |
| **14** | B | 4-$C_5H_4N$ | $C_6H_5$ | | | |
| **15** | B | 2-$C_5H_4N$ | 4-$CH_3$-$C_6H_4$ | **23** | C |  |
| **16** | B | $C_6H_5$ | 4-$NO_2$-$C_6H_4$ | | | |

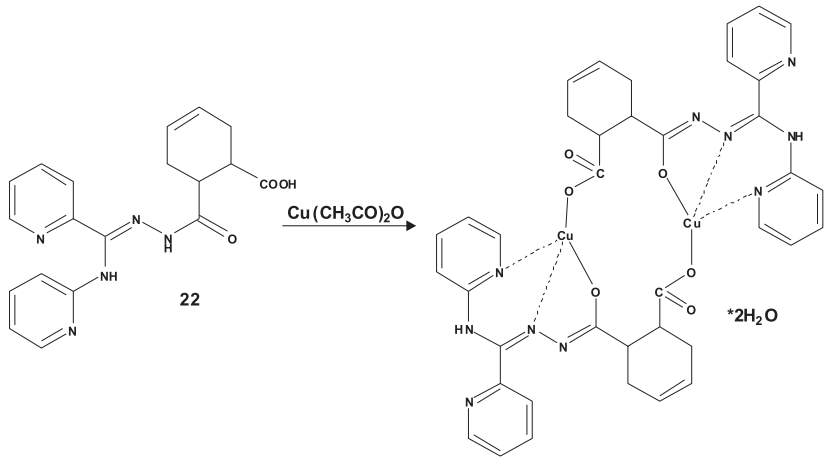



Figure 1. The exemplary reaction of **22** with $Cu^{2+}$ ions leading to formation of copper(II) complex (12)

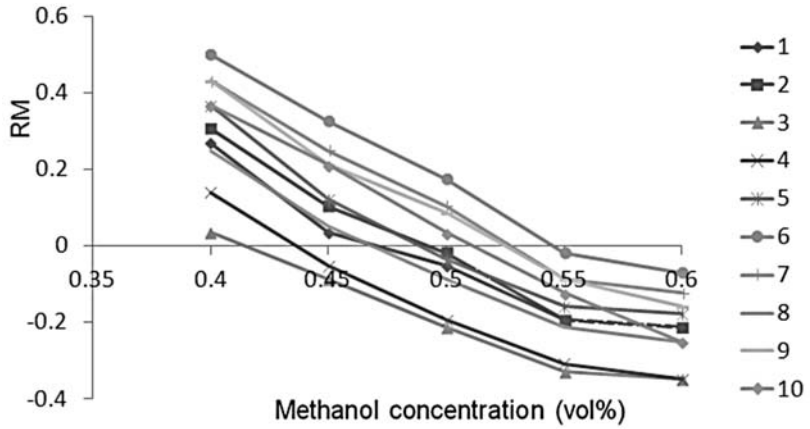



Figure 2. The relationship between $R_M$ values and methanol concentration in the mobile phase of compounds **1-10**

rities and degradation products for all used mobile phases. Compounds **11-23** revealed limited stability in acidic solution - multiple spots occurred on plates after developing in mobile phase with acetic acid addition. Furthermore, chromatograms of compounds **15**, **16** and **23** showed additional spots in methanol-water mobile phases even for fresh dissolved samples. In these cases, unchanged compound was revealed by reaction with copper (II) ions resulting with yellowish spots of created complex on pale blue background. Detection reagent (1% $CuSO_4$ solution in methanol/water mixture) was used basing on reaction described previously (Fig. 2) (12). All linear derivatives **11-23** created color copper(II) complexes in these conditions, while additional spots remained unchanged.

The relative lipophilicity $R_M$ values for five methanol-water mobile phases and compounds **1-23** were calculated by formula $R_M = \log([1-R_f]/R_f)$. The $R_{M0}$ values were calculated from Soczewiński-Wachtmeister equation: $R_M = R_{M0} - S\varphi$, where $\varphi$ was the volume fraction of organic modifier in an aqueous-organic solvent mixture, S was the slope of the regression curve and $R_{M0}$ (lipophilicity index) is the retention parameter for pure water as the eluent. Methanol was chosen as the most recomended organic modifier of the mobile phases for lipophilicity estimation (15).

Results are presented as $R_M$ plots *versus* the percentage composition of methanol in mobile phase (Figs. 2-4). Obtained lipophilicity parameters ($R_{M0}$, S, $\varphi_0$) are presented in Table 2. The $R_{M0}$ values obtained were compared with the theoretical values of partition coefficient ($\log P_{calc}$) calculated by eight available on-line programs (KOWWIN, XLOGP2, XLOGP3, ALOGPS, MLOGP, ALOGP, AC logP, miLogP) (14) (Table 3).

Table 2. The lipophilicity parameters obtained from the linear equation $R_M = R_{M0} - S\varphi$ and correlation coefficients for the investigated compounds.

| Comp. | $R_{M0}$ | S | $\varphi_0$ | $R^2$ |
|---|---|---|---|---|
| **1** | 1.17 | -4.768 | 5.584 | -0.9596 |
| **2** | 1.34 | -5.063 | 6.768 | -0.9748 |
| **3** | 0.82 | -4.393 | 3.588 | -0.9793 |
| **4** | 1.08 | -4.845 | 5.225 | -0.9744 |
| **5** | 1.39 | -5.121 | 7.133 | -0.9567 |
| **6** | 1.67 | -5.354 | 8.934 | -0.9885 |
| **7** | 1.56 | -5.275 | 8.236 | -0.9837 |
| **8** | 1.22 | -4.912 | 5.968 | -0.9750 |
| **9** | 1.58 | -5.343 | 8.427 | -0.9874 |
| **10** | 1.62 | -5.525 | 8.947 | -0.9986 |
| **11** | 1.29 | -2.688 | 3.474 | -0.9959 |
| **12** | 1.42 | -2.528 | 3.601 | -0.9949 |
| **13** | 1.57 | -3.010 | 4.721 | -0.9897 |
| **14** | 1.18 | -2.427 | 2.866 | -0.9842 |
| **15** | 1.63 | -3.118 | 5.090 | -0.9923 |
| **16** | 2.17 | -3.859 | 8.376 | -0.9910 |
| **17** | 1.12 | -2.427 | 2.725 | -0.9803 |
| **18** | 1.43 | -2.523 | 3.618 | -0.9708 |
| **19** | 2.45 | -3.654 | 8.961 | -0.9942 |
| **20** | 1.18 | -2.206 | 2.600 | -0.9708 |
| **21** | 0.96 | -2.208 | 2.111 | -0.9704 |
| **22** | 1.90 | -2.918 | 5.546 | -0.9851 |
| **23** | 1.13 | -2.339 | 2.649 | -0.9777 |

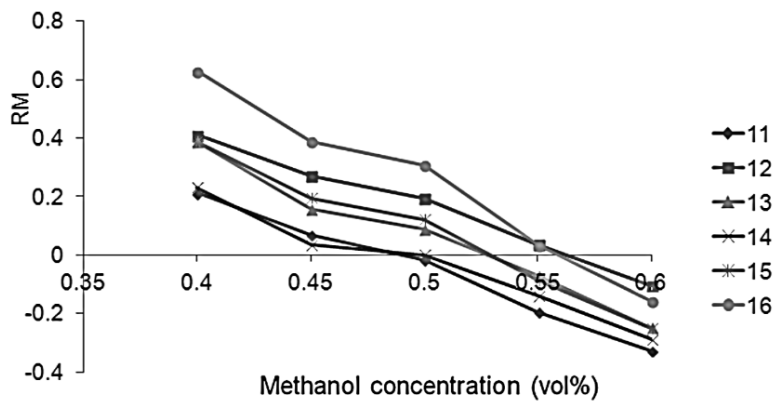



Figure 3. The relationship between $R_M$ values and methanol concentration in the mobile phase of compounds **11-16**

## DISCUSSION AND CONCLUSION

The linear dependence $R_M = R_{M0} - S\varphi$ with high values of correlation coefficients ($R^2$ = 0.97-0.99) was observed for all three groups of compounds in wide range of methanol concentration in mobile phase (40-60%, v/v) which permitted determination of lipophilicity parameters: $R_{M0}$ by extrapolation and $\varphi_0$ by interpolation. Within all analyzed groups were also observed linear relationships of $R_{M0} = f(S)$ with

Table 3. The comparison $R_{M0}$ values of compounds **1-23** with log P values calculated by eight computational programs.

| Comp. | $R_{M0}$ | KOWWIN | XLOGP2 | XLOGP3 | ALOGPS | MLOGP | ALOGP | AC logP | miLogP |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 1.17 | 2.28 | 3.58 | 2.14 | 1.46 | 3.41 | 2.74 | 1.55 | 1.82 |
| **2** | 1.34 | 1.70 | 3.75 | 2.07 | 2.20 | 3.41 | 3.11 | 1.71 | 2.15 |
| **3** | 0.82 | 1.09 | 3.06 | 1.40 | 0.85 | 3.22 | 2.13 | 1.76 | 1.34 |
| **4** | 1.08 | 1.01 | 3.23 | 1.34 | 1.09 | 3.22 | 2.49 | 1.92 | 1.67 |
| **5** | 1.39 | 2.10 | 3.47 | 1.97 | 1.91 | 3.53 | 2.63 | 1.42 | 1.78 |
| **6** | 1.67 | 2.83 | 3.93 | 2.47 | 1.36 | 3.38 | 2.80 | 1.81 | 2.12 |
| **7** | 1.56 | 3.47 | 4.74 | 3.17 | 2.15 | 4.15 | 3.46 | 2.57 | 2.96 |
| **8** | 1.22 | 2.28 | 3.49 | 2.10 | 1.28 | 3.14 | 2.31 | 1.49 | 1.67 |
| **9** | 1.58 | 2.83 | 4.02 | 2.50 | 1.60 | 3.65 | 3.22 | 1.87 | 2.27 |
| **10** | 1.62 | 3.29 | 4.64 | 3.00 | 2.71 | 4.22 | 3.35 | 2.44 | 2.92 |
| **11** | 1.29 | 1.31 | 2.29 | 1.87 | 1.97 | 1.98 | 2.09 | 2.00 | 0.65 |
| **12** | 1.42 | 0.12 | 1.76 | 1.14 | 1.34 | 1.80 | 1.48 | 1.40 | -0.25 |
| **13** | 1.57 | 1.12 | 2.18 | 1.70 | 1.84 | 2.13 | 1.99 | 1.87 | 0.61 |
| **14** | 1.18 | 1.31 | 2.20 | 1.54 | 1.76 | 1.98 | 1.66 | 1.90 | 0.53 |
| **15** | 1.63 | 1.85 | 2.73 | 2.24 | 2.17 | 2.22 | 2.58 | 2.32 | 1.10 |
| **16** | 2.17 | 2.31 | 3.34 | 2.44 | 2.70 | 3.49 | 2.71 | 2.84 | 1.78 |
| **17** | 1.12 | 0.21 | 2.30 | 1.52 | 1.59 | 1.93 | 1.73 | 1.67 | 0.10 |
| **18** | 1.43 | 1.40 | 3.46 | 2.26 | 2.07 | 2.90 | 2.45 | 2.64 | 0.89 |
| **19** | 2.45 | 1.41 | 2.88 | 2.14 | 1.76 | 2.59 | 2.47 | 1.71 | 1.05 |
| **20** | 1.18 | -0.29 | 1.50 | 0.50 | 1.01 | 1.62 | 1.00 | 1.22 | -0.56 |
| **21** | 0.96 | 0.21 | 2.21 | 1.19 | 1.41 | 1.93 | 1.30 | 1.57 | -0.37 |
| **22** | 1.90 | 1.20 | 2.36 | 1.73 | 1.73 | 2.51 | 2.02 | 1.61 | 0.56 |
| **23** | 1.13 | 0.52 | 1.79 | 0.84 | 1.28 | 1.55 | 1.40 | 0.86 | -0.58 |

Table 4. Correlation matrix for various log $P_{calc}$ *versus* $R_{M0}$ relationships within groups ($p < 0.05$, ns – not significant).

| Comp. | KOWWIN | XLOGP2 | XLOGP3 | ALOGPS | MLOGP | ALOGP | AC logP | miLogP |
|---|---|---|---|---|---|---|---|---|
| **1-10** | 0.86 | 0.82 | 0.85 | 0.67 | 0.67 | 0.80 | ns | 0.80 |
| **11-16** | ns | 0.82 | ns | ns | 0.92 | ns | ns | ns |
| **17-23** | 0.76 | ns | ns | ns | ns | ns | ns | 0.80 |

high values of regression ≥ 0.95 which are characteristic for closely related compounds (Fig. 5). This plot confirmed also high structural resemblance between compounds **11-16** and **17-23**.

Molecular mechanism of chromatographic retention is similar for compounds **11-23** and their donor-acceptor properties are also similar to each other. Since retention mechanism in chromatography on reversed phases could be regarded as similar to the ability of diffusion through cell membranes, this suggests their similar bioavailability. The same properties should also be expected between triazoles **1-10**.

Depending on $R_{M0}$ values, compounds could be classified in groups by growing lipophilicity. Since an increase in lipophilicity can be usually connected with the increased biological activity, therefore compounds **19**, **16**, **22** should be expected the most active. Amidst the second group of compounds (**11-16**), there is unexpected $R_{M0}$ value of compound **12** higher than isomers **11** and **14**. For comparison, in the first group, derivatives disubstituted with 2-pyridine (**3**, **4**) showed lower value of lipophilicity index than derivatives possessing 2-pyridine and phenyl rings (**1**, **2**). This phenomenon could be generated by creating intermolecular hydrogen bonds within compound **12** which disguise its hydrophilic groups. On the other hand, compounds **12** and **18** differing in lateral sub-

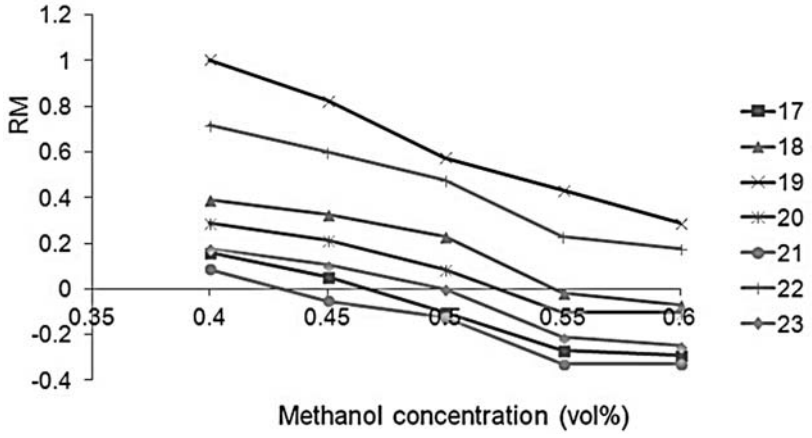



Figure 4. The relationship between $R_M$ values and methanol concentration in the mobile phase of compounds **17-23**

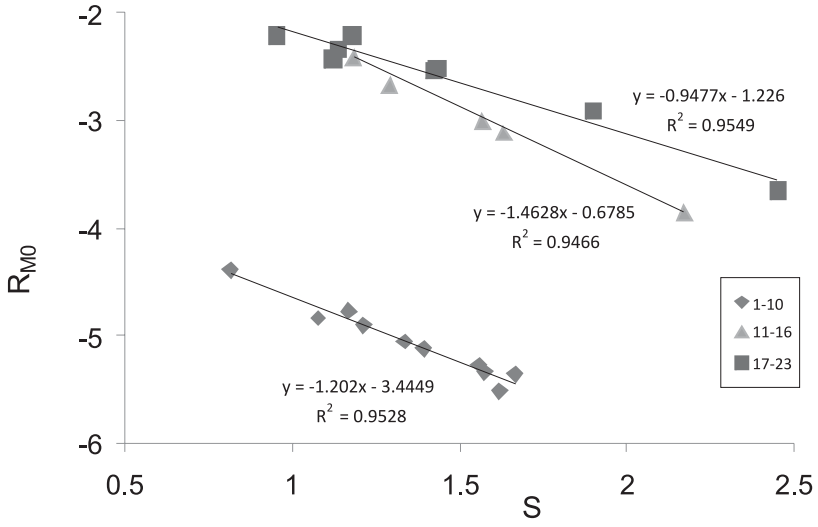



Figure 5. Structural similarity of the analyzed compounds within groups: **1-10** (diamonds), **11-16** (triangles), and **17-23** (squares)

stituent near carboxylic group showed very similar $R_{M0}$ values although all computational methods predicted higher lipophilicity for derivative **18**.

Relative lipophilicity $R_{M0}$ values were compared with log $P_{calc}$ values calculated by computational methods (Table 4). Strong correlations ($R^2 > 0.8$) between log $P_{calc}$ and $R_{M0}$ of compounds **1-10** was found with KOWWIN, XLOGP2, XLOGP3, ALOGP, miLogP programs and moderate ($R^2 \sim 0.67$) with ALOGP and MLOGP. In the second group of compounds (**11-16**), the strongest correlation was obtained by MLOGP, good result was also obtainded by XLOGP2. Only KOWWIN and miLog showed strong significant correlations between calculated and experimental values within third group of compounds (**17-23**).

Computer programs basing on fragmentation method don't consider intramolecular bonds and well-known among amidrazone derivatives tautomerism (15, 16). Five strong correlations were found between log P values calculated by eight programs and lipophilicity indexes of triazole derivatives **1-10**, however only four significant correlations were found for two other groups of acyclic compounds. None of the examined programs showed significant correlations with the values of lipophilicity indexes for three groups of compounds. Therefore, experimentally found lipophilicity

parameters should better reflect the real physicochemical properties of linear $N^3$-substituted amidrazone derivatives than theoretical calculations.

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# APPLICATION OF ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY FOR EXAMINATION OF FREE RADICAL SCAVENGING PROPERTIES OF INSULIN ANALOGS

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**Abstract:** The performed study reflects the innovatory application of EPR spectroscopy which enabled to detect antioxidant properties of insulin analogs. Despite of scientific importance this research indicates the additional benefits from the implementation of insulin therapy in diabetic patients. The two rapid-acting insulin analogs (insulin lispro and insulin aspart), together with two long-acting recombinant analogs of regular human insulin (insulin detemir and insulin lantus) and three recombinant mixtures of analogs (biphasic insulin lispro 25/75 – BILis 25, biphasic insulin aspart 30/70 – BIAsp 30, biphasic insulin aspart 50/50 – BIAsp 50) were examined by X-band (9.3 GHz) electron paramagnetic resonance (EPR) spectroscopy. The kinetics of insulin interactions with free radicals was determined. The antioxidative insulin properties were characterized by the ability to quench free radicals. As a result, the decrease of EPR signal of the free radical reference appears. DPPH (1,2-diphenyl-2-picrylhydrazyl) was used as the paramagnetic reference in this study. The four groups of insulins in terms of interactions with free radicals were found and their interactions with DPPH decreased as follows: I group (rapid-acting insulin analog, insulin lispro, analog mixtures, BIAsp 50, BIAsp 30) > II group (rapid-acting insulin analog, insulin aspart) > III group (analog mixtures, BILis 25) > IV group (long-acting insulin analogs, insulin detemir, insulin lantus). In conclusion, insulin interactions with free radicals depend on the type of insulin.

**Keywords:** insulin analogs, antioxidant, free radicals, DPPH, EPR spectroscopy

Insulin analogs are commonly used in intensive antidiabetic therapy. Insulin is mainly used in type 1 diabetes mellitus as well as in type 2 diabetic patients as an add-on therapy to the non-insulin antidiabetic medications (1, 2). The rapid-acting human insulin analogs have a more rapid subcutaneous absorption, faster onset and shorter duration of action than regular human insulin. Their use improves postprandial glycemic control and decreases the risk of hypoglycemia (3). The long-acting analogs of insulin are more stable in the solution and have a longer half-life than neutral protamine Hagedorn (NPH) insulins. Moreover, the mentioned analogs are characterized by a relatively flat action profile, which reduces variability of glycemic values and decreases the incidence of hypoglycemia. In consequence, the long acting insulin analogs provide a more effective, safer and physiologic insulin replacement therapy in comparison with NPH insulins (4, 5). The therapy with premixed insulin analogs is one of the possible models of intensive insulin therapy (6) providing basal and postprandial coverage with lower number of daily injections (7). When compared with basal insulin analogs, premixed insulin analogs demonstrate higher reduction of $HbA_{1C}$ value (8). Despite the fact that all of the above mentioned therapeutic agents belong to homogenous pharmacological category of insulin analogs, their molecular structure and composition as well as physicochemical properties are diverse, which may influence the efficacy of the management process (9). The presence of free radicals in insulin samples could modify their pharmacodynamics and also pharmacokinetic properties.

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Therefore, the aim of this study was to determine the interactions between different types of insulins and free radicals in terms of their antioxidative properties. Electron paramagnetic resonance (EPR) spectroscopy was used to observe the insulin effect on unpaired electrons in a model free radical source such as DPPH. The performed spectroscopic analyzes were innovatory. The EPR tests with DPPH were already applied both in pharmacy (10-12) and medicine (13-15), but they have not been conducted for insulins so far. It is proposed to apply the EPR results practically.

## EXPERIMENTAL

### Studied samples

The following insulins were examined: the rapid-acting insulin analogs – insulin lispro (Humalog®: 100 U/mL, Eli Lilly and Co.), and insulin aspart (NovoRapid®: 100 U/mL, Novo Nordisk); the long-acting insulin analogs – insulin detemir (Levemir®: 100 U/mL, Novo Nordisk) and insulin glargine (Lantus®: 100 U/mL, Sanofi-Aventis); the premix insulin analogs – insulin premix containing 30% soluble insulin aspart and 70% protamine-crystallized insulin aspart – BIAsp 30 (NovoMix 30®: 100 U/mL, Novo Nordisk), insulin premix containing 50% soluble insulin aspart and 50% protamine-crystallized insulin aspart – BIAsp 50 (NovoMix 50®: 100 U/mL, Novo Nordisk), insulin premix containing 25% insulin lispro solution and 75% insulin lispro protamine suspension – BILis 25 (Humalog Mix 25®: 100 U/mL, Eli Lilly and Co.).

### EPR measurements

Interactions of the tested insulins with free radicals were examined by the use of electron paramagnetic resonance spectroscopy with microwaves of frequency of 9.3 GHz from an X-band. DPPH (1,2-diphenyl-2-picrylhydrazyl) as the paramagnetic reference was the model source of free radicals. Free

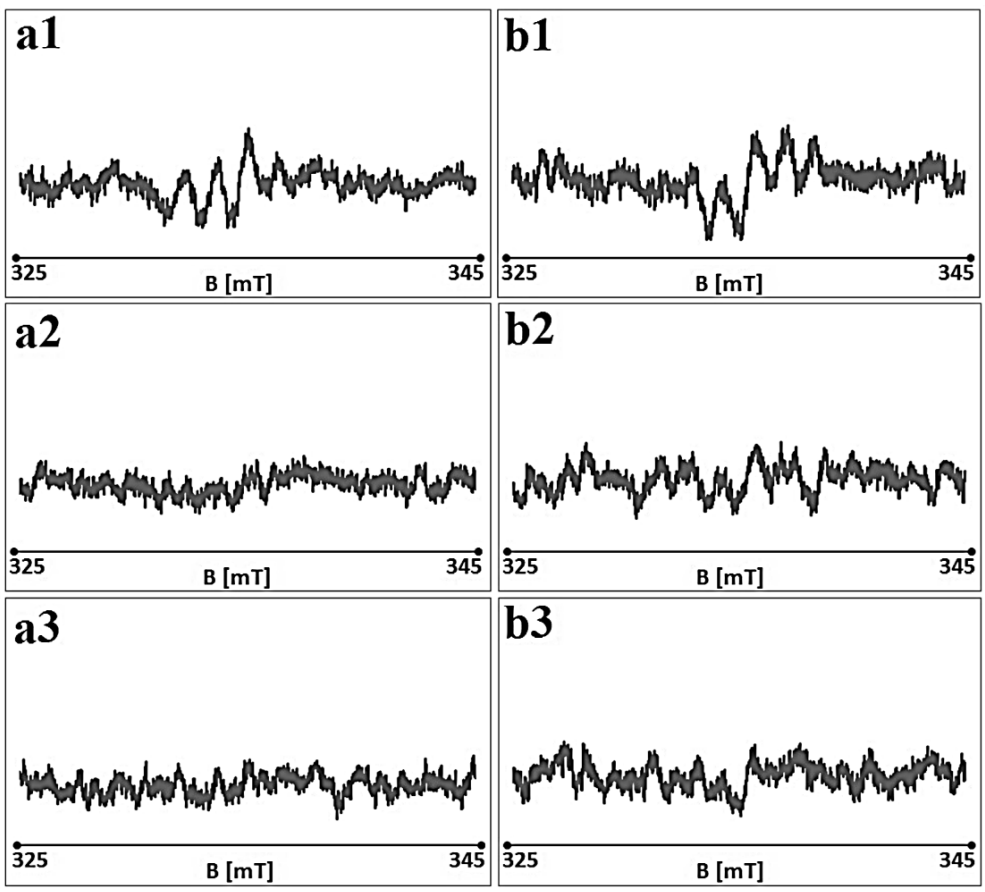


Figure 1. EPR spectra of DPPH free radicals interacting with the fast interacting insulin analogs, insulin lispro (a1, a2, a3), and insulin aspart (b1, b2, b3), during 5 min (a1, b1), 30 min (a2, b2) and 60 min (a3, b3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

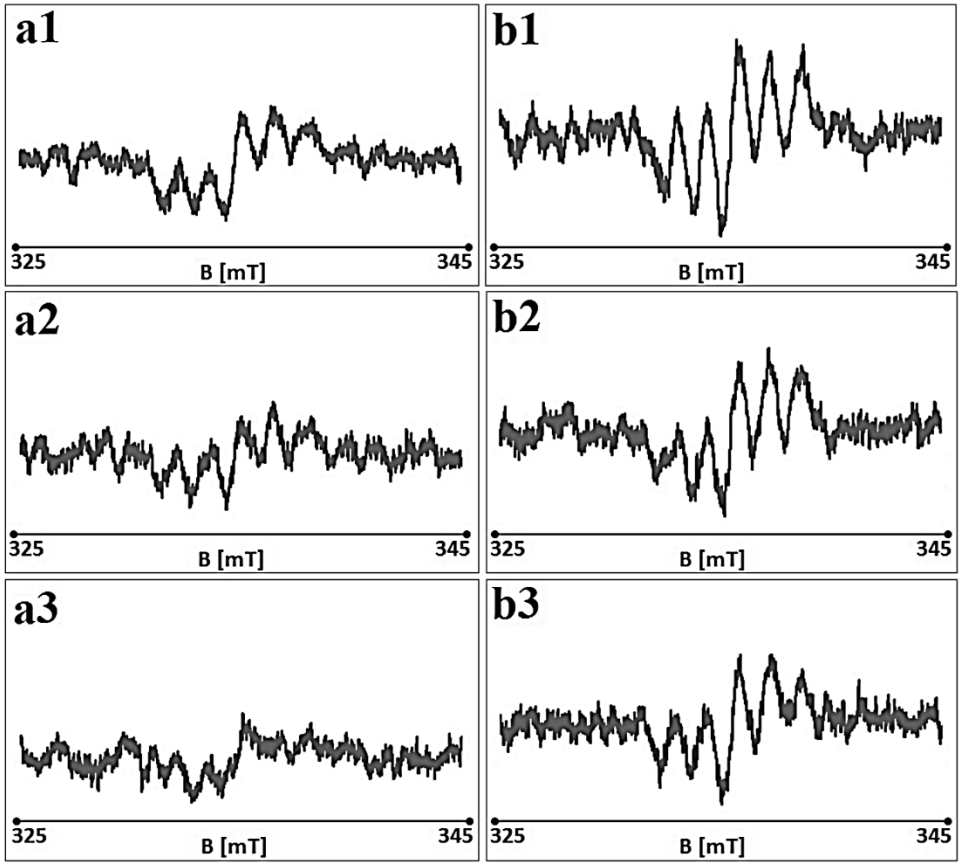


Figure 2. EPR spectra of DPPH free radicals interacting with human analogs insulin, detemir (a1, a2, a3), and glargine (b1, b2, b3), during 5 min (a1, b1), 30 min (a2, b2) and 60 min (a3, b3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

radicals with unpaired electrons located on nitrogen atoms existed in this reference (16, 17). The samples of DPPH in 10% ethyl alcohol solution were prepared and the EPR line of the model free radicals was measured. Next, the EPR line of DPPH in contact with the tested insulin was measured. The EPR line of DPPH was compared with EPR lines of DPPH which interacted with the tested insulins. As the result of interactions of DPPH and insulin the EPR line of DPPH decreased. This decrease of spectral line was the effect of decreased number of unpaired electrons in the used model of free radicals – DPPH, which stood for the decreased number of free radicals in it. The antioxidative properties of the tested insulins are proportional to quenching of free radicals and the EPR signals of DPPH. The quenching of free radicals by insulins is responsible for the decrease of amplitude (A) of EPR line of DPPH. In our measurements, the amplitude (A) was the most important parameter in achieving the aim of the analysis. In EPR spectroscopy, the absorption of microwaves by paramagnetic sample was measured (16, 17). Free radicals of DPPH absorbed microwaves, therefore, unpaired electrons of the model free radicals were excited to the higher energy levels. This energy was returned by unpaired electrons in relaxation processes and the electrons came back to the lower energy levels. Free radicals of DPPH were quenched by interactions with insulin and the lower number of unpaired electrons were excited by microwaves to the higher energy levels, while the lower EPR signals for DPPH were observed. The tested samples (DPPH and insulins) were placed in the thin-walled glass tubes with the external diameter of 1 mm. The empty tubes were free of EPR signals.

EPR spectra of DPPH were numerically detected with electron paramagnetic resonance spectrometer with magnetic modulation of 100 kHz produced by RADIOPAN (Poznań, Poland) and the RAPID

SCAN UNIT of JAGMAR (Kraków, Poland). Microwave frequency was measured by MCM101 recorder of EPRAD (Poznań, Poland). EPR spectra of DPPH and DPPH interacting with insulins, were measured as the first-derivative lines. The acquisition of the individual EPR line was done during the time of 1 second. The EPR spectra of DPPH were measured with the low microwave power of 2.2 mW to avoid microwave saturation of the resonance curves. The total microwave power produced by klystron of the EPR spectrometer was 70 mW. The microwave power of 2.2 mW corresponded to the attenuation of 15 dB at the exit. The following parameters of the EPR spectra of DPPH were determined, amplitudes (A), and g-factors. Amplitudes (A) of the EPR spectra increased with increasing amount of free radical contents in the samples (16, 17). The most important for DPPH are dipolar interactions between free radicals which broaden EPR lines. The g-factor of free radicals of DPPH was determined from the electron paramagnetic resonance condition in the following way (16, 18):

$$g = h\nu/\mu_B B_r,$$

where: h – Planck constant, ν – microwave frequency, $\mu_B$ – Bohr magneton, $B_r$ – induction of resonance magnetic field. The g-factor depends on localization of unpaired electrons in the sample (17, 18).

The accuracy of amplitude (A) of EPR line of DPPH was ± 0.01 a.u. The microwave frequency (ν) and the induction of magnetic field (B), were measured with the accuracy of ± 0.0002 GHz, and ± 0.01 mT, respectively. The accuracy of g-value equalled ± 0.0002. The errors for the spectral physical values were determined by the use of total differential method. Total differential takes into account the errors of all the factors affecting the determined value. Differential of the function contains its derivatives with respect to all factors.

The experimental errors for the parameters of EPR spectra were very low, because the parameters of the EPR spectra of DPPH were analyzed by the professional spectroscopic programs of JAGMAR (Kraków, Poland) and LabVIEW 8.5 of National Instruments (USA).

## RESULTS AND DISCUSSION

Free radicals are key factors responsible for the development of diabetic vascular complications. The mechanisms underlying the diabetic endothelial dysfunction may involve several biochemical pathways with an increase in glucose concentration providing the initial metabolic disorders (19, 20). Potential mechanisms by which hyperglycemia can lead to excessive free radical formation include direct autoxidation of glucose, activation of lipooxygenase, stimulation of glycation pathways, activation of kinase C activity, intracellular activation of

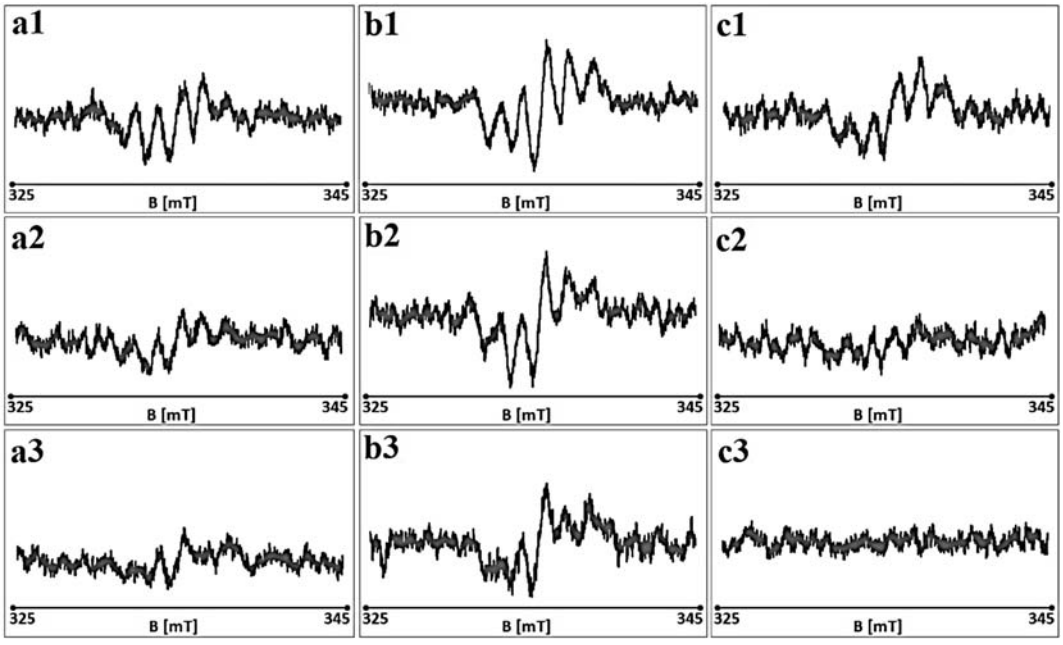


Figure 3. EPR spectra of DPPH free radicals interacting with mixed type insulin, BILis 25 (a1, a2, a3), BIAsp 30 (b1, b2, b3), and BIAsp 50 (c1, c2, c3), during 5 min (a1, b1, c1), 30 min (a2, b2, c2) and 60 min (a3, b3, c3). **B** is the magnetic induction of the field produced by electromagnet of the EPR spectrometer

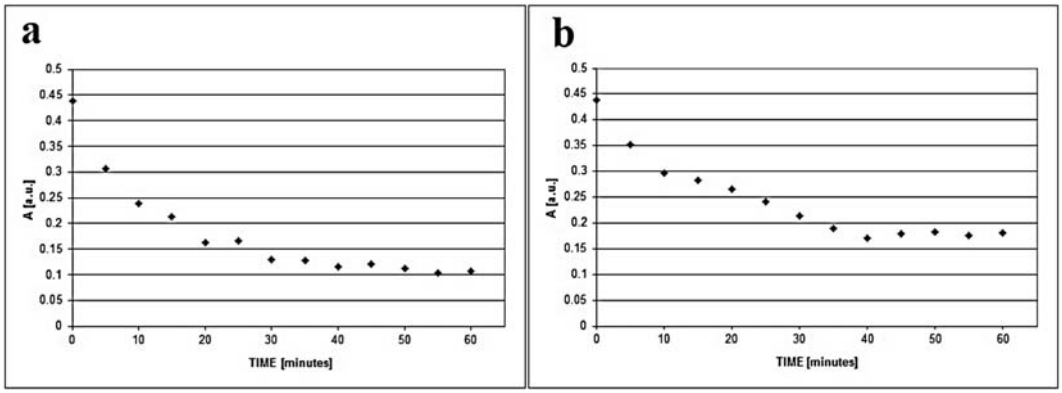


Figure 4. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with the fast interacting insulin analogs, insulin lispro (a), and insulin aspart (b), with time of interaction (t)

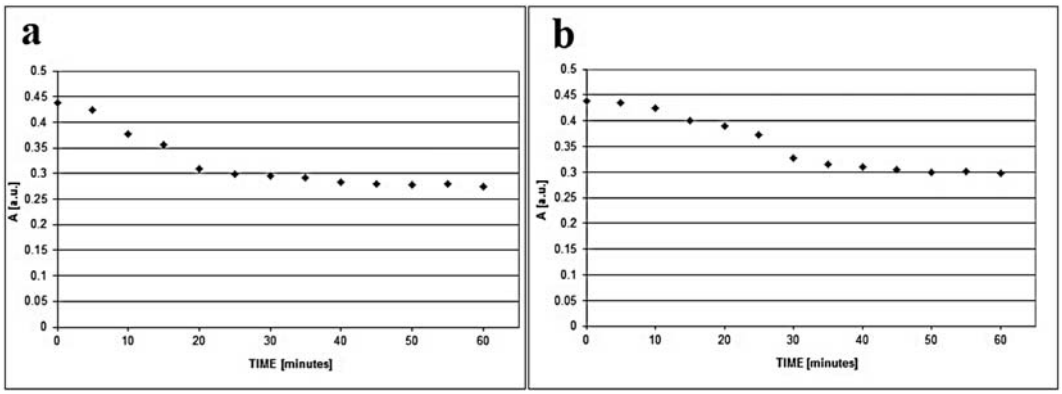


Figure 5. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with human analogs insulin, insulin detemir (a), and insulin glargine (b), with time of interaction (t)

sorbitol pathways as well as activation of NADPH oxidases (21). Moreover, the decreased potential of the extracellular and intracellular antioxidant capacity has been reported as a factor responsible for enhancing the oxidative state in diabetic patients (22). The experimental and clinical studies have revealed that excessive free radicals expression trigger insulin resistance, independently of the presence of other risk factors, such as obesity, impaired fasting glucose and metabolic syndrome (23, 24). Our present study has indicated that all the applied insulin analogs interact with free radicals and demonstrate antioxidant properties *in vitro*. The quenching of free radicals by insulins was observed as a decrease of amplitude (A) of the EPR lines of the reference – DPPH with g-factor of 2.0036 (Figs. 1-6).

DPPH was a well-defined free radical reference. Electron paramagnetic resonance (EPR) spectroscopy was used as the sensitive physical method to examine interactions of the sample with free radicals. Decrease of the EPR signal of paramagnetic DPPH indicated unmistakable interactions of the sample with free radicals. For the samples which do not interact with free radicals EPR signal of DPPH remains unchanged. The obtained g-value (2.0036) confirmed the chemical purity of the reference sample. Unpaired electrons exist in its structure on nitrogen (N) atoms. The interactions of unpaired electrons with the magnetic moment of nitrogen nuclei and structure of the energy levels in DPPH, decided about the g-values. The measured g-value comply with the theoretical one for DPPH, which confirmed that the model was properly used (17). The advantages of DPPH as the paramagnetic model in examination of insulin interactions with free radicals were a stabile chemical structure and a strong EPR line. The changes of the clearly visible EPR signal of DPPH after interactions with insulins were easy to determine, therefore we were able to achieve

the high accuracy of our measurements. The accuracy of the used EPR spectrometer and the additional equipment (recorder of microwave frequency, and NMR magnetometer) played also an important part in obtaining precise results. The quenching of the EPR lines of DPPH decreased with the time of interactions with particular insulins, finally being saturated. EPR spectra of DPPH in contact with the analyzed analogs of insulins during 5, 30, and 60 min are shown in Figures 1-3. The EPR signals of free

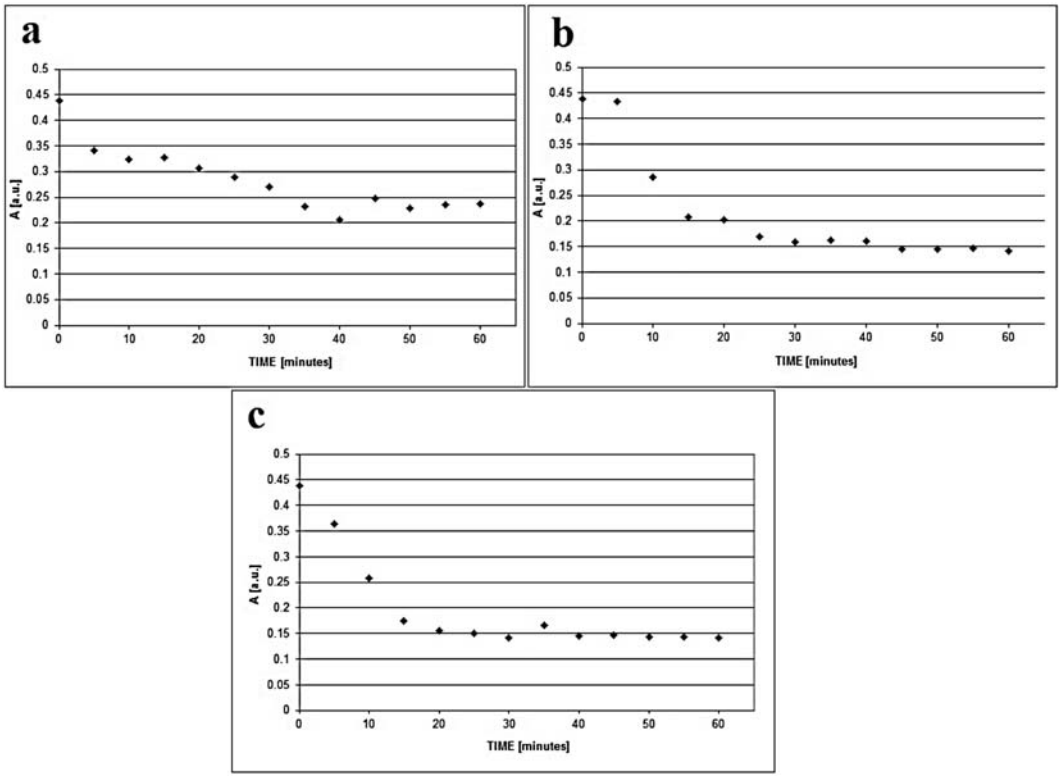


Figure 6. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with mixed type insulin, BILis 25 (a), BIAsp 30 (b), and BIAsp 50 (c), with the time of interaction (t)

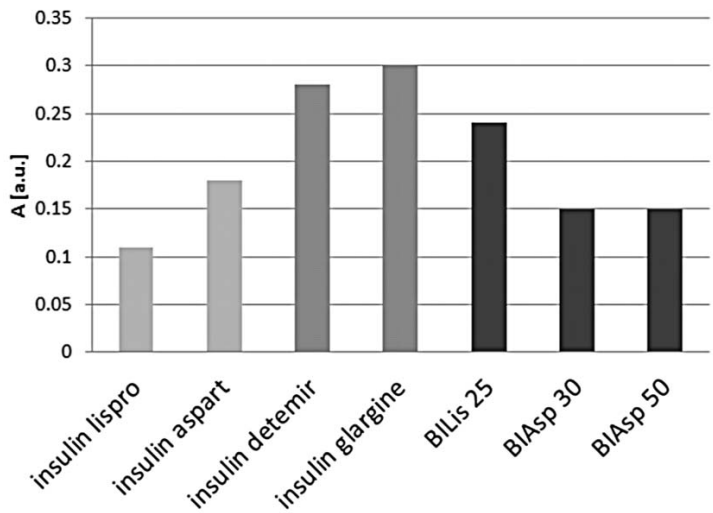


Figure 7. Comparison of the amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH free radicals interacting with the tested insulins after 60 min of interaction

radicals of DPPH decrease with increasing time. The amplitudes (A) of the EPR spectra of DPPH interacting with the examined insulins *versus* the time of interactions are presented in Figures 4-6. Amplitudes (A) of EPR lines of DPPH in contact with the particular insulins during 60 min are compared in Figure 7.

Taking the quenching of free radicals into account (Figs. 1-7), the insulins from the following four groups (I-IV) differ in amplitudes (A) [a.u. – arbitrary units] of DPPH EPR lines, I group, A, 0.11-0.15 a.u. (fast interacting analogs, insulin lispro, analog mixtures, BIAsp 50, and BIAsp 30); II group, A, 0.18 a.u. (fast-acting analogs, insulin aspart); III group, A, 0.24 a.u. (analog mixtures, BILis 25); IV group, A, 0.27-0.30 a.u. (long-acting insulin analogs, insulin detemir, insulin glargine).

The interactions of insulin samples with free radicals decrease as follows: I group (A, 0.11-0.15 a.u.) > II group (A, 0.18 a.u.) > III group (A, 0.24 a.u.) > IV group (A, 0.27-0.30 a.u.).

The antioxidative properties of the examined insulins decrease in a similar way to the presented relation for quenching of free radicals and amplitudes (A) of the reference - DPPH. The strongest interactions with free radicals were observed for the I group of insulin (fast interacting analogs, insulin lispro, analog mixtures, BIAsp 50, and BIAsp 30), because the lowest amplitudes (A, 0.11-0.15 a.u.) of EPR lines of DPPH in contact with these samples were obtained. The weakest interactions with free radicals were observed for the IV group of insulin (long-acting insulin analogs, insulin detemir, insulin glargine), because of the highest (A, 0.27-0.30 a.u.) EPR lines of DPPH in contact with these analyzed active substances.

The analysis of the interaction time of the insulins with free radicals pointed out the differentiation in the tested groups. The times after the amplitudes (A) of EPR lines in the following groups decreased to the constant values in the following manner: I group, ($t_{min}$, 20-40 min) (fast-acting insulin analogs, insulin lispro (40 min); analog mixtures, BIAsp 50 (20 min), and BIAsp 30 (30 min); II group, ($t_{min}$, 40 min) (fast-acting analogs, insulin aspart (40 min); III group, ($t_{min}$, 40 min) (analog mixtures, BILis 25 (40 min); IV group, ($t_{min}$, 35-40 min) (long-acting insulin analogs, insulin detemir (35 min), insulin glargine (40 min).

In the I group of insulins with the strongest antioxidative properties, fast interactions with free radicals were observed for two drugs, BIAsp 50 ($t_{min}$ = 20 min) and BIAsp 30 ($t_{min}$ = 30 min). The relatively slow interactions with free radicals were obtained for insulin lispro ($t_{min}$ = 40 min). In the II group of insulins slow interactions with free radicals existed. The long time ($t_{min}$) was revealed in the case of insulin aspart (40 min). In the III insulins group slow interaction with free radicals (BILis 25 ($t_{min}$ = 40 min)) was visible. In the IV group of insulins, insulin detemir ($t_{min}$ = 35 min) interacted relatively faster with free radicals than insulin glargine ($t_{min}$ = 40 min).

Taking into consideration all the examined insulins, relatively faster interactions with free radicals were obtained for BIAsp 50 ($t_{min}$ = 20 min) and BIAsp 30 ($t_{min}$ = 30 min).

The obtained results confirmed usefulness of an X-band (9.3 GHz) electron paramagnetic resonance spectroscopy in determining the interactions of insulins with free radicals. The application of DPPH as the paramagnetic reference in this study was chosen. This reference contained high amount of free radicals and strong EPR signals were measured for it, therefore, the quenching of its resonance line by insulin samples was clearly visible. Professional spectroscopic programs additionally affected the accuracy of the results. The decrease in values of the amplitude of DPPH EPR line and the kinetics of the changes with increasing time of DPPH – the insulin interactions illuminated the antioxidative properties of insulins.

EPR spectroscopy and model free radical DPPH sample were earlier documented in our previous study as an useful tool for assessing free radical expression and antioxidant properties of the biosynthetic human insulins of three groups: short-acting, intermediate-acting, and pre-mixed insulins (25).

## CONCLUSIONS

Electron paramagnetic resonance studies of different insulins interacting with model free radicals proved that:

All the insulins actively interact with free radicals, and the quenching of DPPH free radicals was observed.

Interactions with free radicals, which resulted in quenching the EPR line of DPPH, depend on the type of insulin, and, therefore, the four insulin groups with different antioxidative properties were distinguished, I group (fast-acting analog, insulin lispro, analog mixtures, BIAsp 50, BIAs*p* 30); II group (fast-acting analog, insulin aspart); III group (analog mixture, BILis 25); IV group (long-acting analogs, insulin detemir, insulin glargine).

The interactions of insulins with free radicals resulted in the quenching amplitudes (A) of EPR

line of DPPH decrease in the following order, I group (A, 0.11-0.15 a.u. > II group (A, 0.18 a.u. > III group (A, 0.24 a.u. > IV group (A, 0.27-0.30 a.u.).

The insulins differ in time of interactions with free radicals. The relatively faster interactions with free radicals were characteristic for BIAsp 50, and BIAsp 30.

The usefulness of electron paramagnetic resonance spectroscopy and model free radical reference – DPPH in determining the interactions of insulins with free radicals was confirmed.

The results of our pioneering research revealing the antioxidative properties of insulin analogs should be carefully assessed and confirmed in human clinical studies. Moreover, future direction in EPR study concerning the interactions between free radicals and insulins should be related to the assessment of the influence of UV, storage time and storage temperature on free radicals' expression in opened vialled insulin samples. It may be of great importance in the verification of currently recommended storage conditions of punctured insulin vials used in different types of insulin delivery devices including pens, jet injectors and external insulin pumps.

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# THE EFFECT OF β-CYCLODEXTRIN ON THE RESOLUTION OF FREE AND CONJUGATED FORMS OF DEOXYCHOLIC AND CHENODEOXYCHOLIC ACIDS BY TLC-DENSITOMETRY

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**Abstract:** This paper describes a method for the separation of two optically active bile acids, namely deoxycholic and chenodeoxycholic and their conjugated forms with glycine and taurine by one dimensional (1D) reversed phase system (RP-TLC and RP-HPTLC) and also on HPTLC chiral plates. Different chromatographic plates and mobile phase systems were tested in this work. The spots were detected with 10% ethanolic solution of sulfuric acid, followed by densitometric measurements at 400 nm. The best results of separation of difficult to separate pair of deoxycholic and chenodeoxycholic acids were achieved by 1D RP-HPTLC technique on chromatographic plates RP-2$F_{254}$ developed with methanol-water-β-cyclodextrin (3%) in volume composition 40 : 10 : 5. Additionally, the influence of β-cyclodextrin concentration (in the range of 1 to 5%) on the separation of studied bile acids and their conjugates with glycine and taurine was estimated. The developed method proved to be selective, robust and less time-consuming than 2D development reported in literature for the separation and identification of optically active free and also conjugated forms of deoxycholic acid and chenodeoxycholic acid. The proposed separation system may be useful as a preparative step in the medical or pharmaceutical analysis of studied bile acids by advanced chromatographic methods, such as HPLC or GC.

**Keywords:** bile acids, β-cyclodextrin, optically active compounds, TLC, densitometry

Deoxycholic (DC) and chenodeoxycholic (CDC) acids are typically C24 polyhydroxy steroids which are formed in the liver from cholesterol. They serve different functions, such as elimination of cholesterol from the body and emulsifying lipids and fat-soluble vitamins in the intestine to form micelles that can be transported *via* the lacteal system (1). The conjugated bile acid salts with taurine or glycine, respectively, are more efficient especially at emulsifying fats. Determination of bile acids concentration in biological samples (e.g., serum) provides information important in clinical study, for instance in diagnosis of hepatobiliary diseases. The analysis of bile acids including DC and CDC in biological matrices as well as in pharmaceutical samples depends on accurate sample pretreatment. Among various advanced chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) is applicable to separation of bile acids (as free and conjugates with taurine and glycine) in preliminary study of bile acid mixture. It is well known that thin-layer chromatography coupled with densitometry is a fast, simple and inexpensive method as opposed to other chromatographic techniques. The main advantage of TLC-densitometry is that a number of bile acids can be handled simultaneously in one step. In addition to this, the bile acids analyzed by TLC with densitometric detection can be visualized directly on chromatographic plate after removing off mobile phase. However, in the case of TLC analysis of described bile acids, the scientists must be confronted with a problem of separation of two optically active bile acids DC, CDC and their conjugates. Literature review indicates that in order to overcome the problem of TLC separation of different classes of optically active compounds, various chiral selectors as impregnating reagents (stationary phase modifiers) as well as mobile phase additives have been used (2-7). Of many chiral selectors which have been presented in the available literature, β-cyclodextrin (β-CD) is often recommended as mobile phase additive or stationary phase modifier for the resolution of

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selected optically active compounds (medical and also pharmaceutical importance), like, for example: propranolol and atenolol (8), ropivacaine (9), mandelic acid (10), some amino acids (11) or cefaclor epimers (12).

According to the best authors knowledge, only few papers have reported the use of β-cyclodextrin for the separation of difficult to separate pair of bile acids: DC and CDC. These studies involved mainly the two dimensional (2D) reversed phase (RP) high performance thin-layer chromatographic technique (HPTLC) and silica gel RP-18$F_{254}$ as stationary phase (13-15). The present work reports the direct resolution of DC and CDC and their conjugated forms with glycine and taurine by means of β-cyclodextrin as mobile phase additive in one dimensional (1D) RP-HPTLC system coupled with densitometry. Different chromatographic plates have been tested in this study, such as RP-2$F_{254}$, RP-8$F_{254}$ and RP-18$F_{254}$. Effect of variation of β-cyclodextrin concentration in mobile phase: methanol-water was studied to optimize the chromatographic conditions, appropriate for complete bile acid resolution. The results of studies obtained on RP-HPTLC plates were compared with those obtained on HPTLC chiral plates.

## EXPERIMENTAL

### Chemicals and sample preparations

The reference standards of deoxycholic acid (DC), chenodeoxycholic acid (CDC), and also their conjugated forms as sodium salts, such as taurodeoxycholic acid (TDC), taurochenodeoxycholic acid (TCDC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard mixture containing 5 mg of each studied bile acid in 1 mL was prepared in methanol (POCh, Gliwice, Poland). β-Cyclodextrin (β-CD, = 98%) was from Sigma-Aldrich. Aqueous solutions of β-CD in the following concentrations: 0.5, 1, 2, 3 and 5% were obtained by dissolving of proper amount of this substance in water at higher temperature. The following components of mobile phase: methanol for liquid chromatography (E. Merck, Darmstadt, Germany), and distilled water (Merck-Millipore, Molsheim, France) were used in this work. Sulfuric acid min. 95% from POCh (Gliwice, Poland) was applied to prepare ethanolic solution of sulfuric acid as visualizing reagent at concentration 10%.

### Materials

The following reversed phase plates for RP-TLC and RP-HPTLC analysis were used: glass plates RP-8$F_{254}$ (E. Merck, Art. 1.15684), glass plates RP-2$F_{254}$ (E. Merck, Art. 1.13726), aluminum plates RP-18$F_{254}$ (E. Merck, Art.1.05559) and glass HPTLC chiral plates (E. Merck, Art. 14101).

### Measuring optical rotation of examined bile acids

In order to confirm the optical activity of examined bile acids: DC, CDC, GDC, GCDC, TDC and TCDC, which is not widely described for these compounds in literature, digital polarimeter type P-2000 from Jasco (United Kingdom) was applied. Optical activity of six investigated bile acids, which is the measure of the ability of them to rotate plane polarized light, was expressed as specific rotation ($\alpha_D^{22}$). For measuring this parameter for all studied bile acids at 22 ± 1°C, the methanolic solutions of respective compound at concentrations 10 mg/mL were used. The length of the polarimeter tube was 5 cm. The source of light was sodium lamp.

### Chromatography

Chromatography was performed on 10 × 10 cm reversed phase plates: RP-18$F_{254}$, RP-2$F_{254}$, RP-8$F_{254}$, and also on HPTLC chiral plates. Samples of mixture containing six examined bile acid: DC, CDC, GDC, GCDC, TDC and TCDC in quantity of 5 µL were applied on the plates.

The chromatograms were developed at 18 ± 2°C in a 10 × 20 cm chromatographic chamber (Camag, Switzerland) which has been previously saturated with solvent vapors during 30 min. The development distance was 8 cm. After developing with methanol-water (40 : 10, v/v) or methanol-water-β-CD (40 : 10 : 5, v/v/v), respectively, the chromatographic plates were dried at 18 ± 2°C using a fume cupboard. Visualization of obtained spots was conducted by dipping the plates in 10% ethanolic solution of sulfuric acid and next, heating them at 110 ± 2°C for 15 min. The plates were scanned by Camag TLC Scanner 3 (Muttenz, Switzerland) which was controlled by WinCATS 1.4.2 software. All densitometric measurements were conducted in reflectance absorbance mode at wavelength of 400 nm. This wavelength was optimal for the examined bile acids and hence it has been selected for further densitometric analysis. The source of radiation was a deuterium lamp. The scanning speed was 20 nm/s and the data resolution was 100 µm/step. The slit dimension was kept at 8.00 × 0.40 mm, Macro. Each analysis was repeated three times. Densitograms obtained under described conditions allowed to estimate the efficiency of separation of six examined bile acids from their mixture.

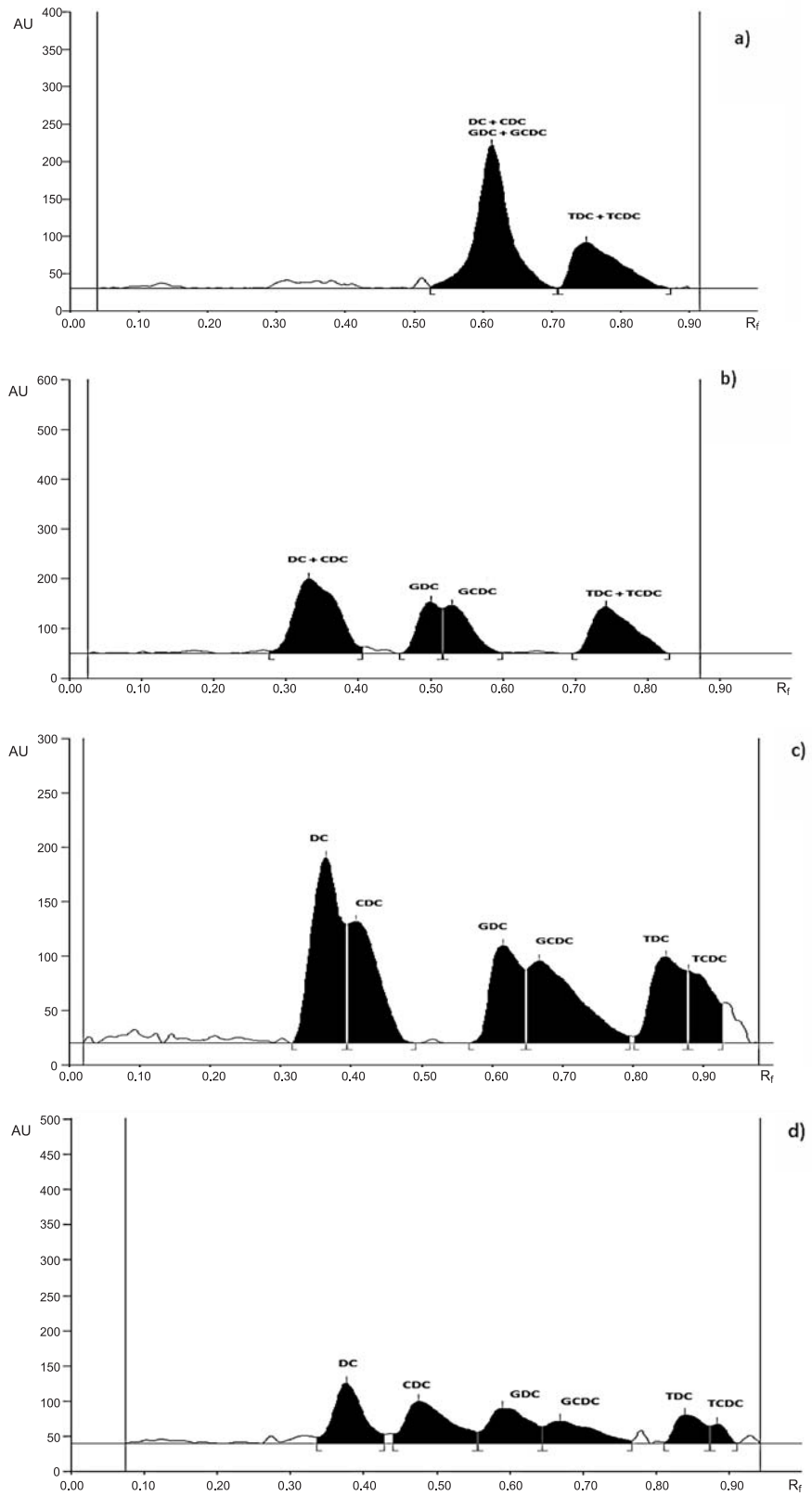


Figure 1. Chromatograms showing the resolution of examined bile acids on reversed phase plates RP-$2F_{254}$ developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-β-CD (0.5%) in volume composition 40 : 10 : 5 (b), methanol-water-β-CD (1%) in volume composition 40 : 10 : 5 (c) and methanol-water-β-CD (3%) in volume composition 40 : 10 : 5 (d)

## RESULTS AND DISCUSSION

The purpose of the work reported herein was to develop a simple and rapid TLC-densitometric method suitable for the complete separation and characterizing of two optically active bile acids: deoxycholic and chenodeoxycholic and their conjugates with taurine and glycine (DC, CDC, TDC, TCDC, GDC, GCDC). Optical activity of discussed compounds confirmed the polarimetric measurements. For examined bile acids, the following values of $\alpha_D^{22}$ were obtained: +61.2° (DC), +15.4° (CDC), +41.2° (TDC), +22.4° (TCDC), +50.2° (GDC) and +13.4° (GCDC). During preliminary study, various chromatographic conditions (different adsorbents and mobile phases) in normal and reversed phase systems (NP-TLC and RP-TLC) were tested. In order to improve the effect of bile acids separation by NP-TLC technique on silica gel 60 and 60$F_{254}$, one of the most popular chiral selector, namely β-cyclodextrin (β-CD) was applied. Aqueous solution of β-CD was used as mobile phase additive and also as impregnating agent of silica gel (stationary phase). Unfortunately, the applied chromatographic conditions in NP-TLC system consisted of silica gel impregnated with aqueous solution or aqueous-methanol solution of β-CD, respectively, could not provide complete resolution of examined bile acids including unconjugated DC and CDC. Similarly, none of prepared mobile phases containing β-CD in various proportions, such as methanol-water-β-CD, methanol-acetic acid-β-CD, chloroform-methanol-acetic acid-β-CD, methanol-acetic acid-water-β-CD, which have been tested in preliminary study, did not allow complete separation of studied bile acids. Efforts were continued, and in further steps of this experiment it was decided to use one dimensional (1D) reversed phase system (RP-TLC, RP-HPTLC) and also HPTLC chiral plates. In order to achieve satisfactory resolution of two investigated bile acids (DC, CDC) and their conjugates, different chromatographic plates from E. Merck (RP-18$F_{254}$, RP-2$F_{254}$, RP-8$F_{254}$, chiral plates) and mobile phase consisting of methanol-water-β-CD in volume composition: 40 : 10 : 5 were used. In this paper, the influence of β-CD content in mobile phase applied and the type of chromatographic plates on the separation of studied bile acids and their conjugates with glycine and taurine, respectively, by means of 1D RP-HPTLC and also 1D RPTLC system are described.

The first of RP-HPTLC chromatographic systems which has been tested, was consisted of RP-2$F_{254}$ plates as stationary phase and methanol-water-β-CD as mobile phase in volume composition 40 : 10 : 5. Different concentration in [%, w/w]: 0.5; 1; 2; 3 and 5 of aqueous solution of β-CD added to mobile phase was applied. The results of analyzed mixture obtained using RP-2$F_{254}$ plates and mobile phase containing β-CD at different concentration indicate that the use of RP-2$F_{254}$ plates and methanol-water-β-CD (40 : 10 : 5, v/v/v) improves the resolution of

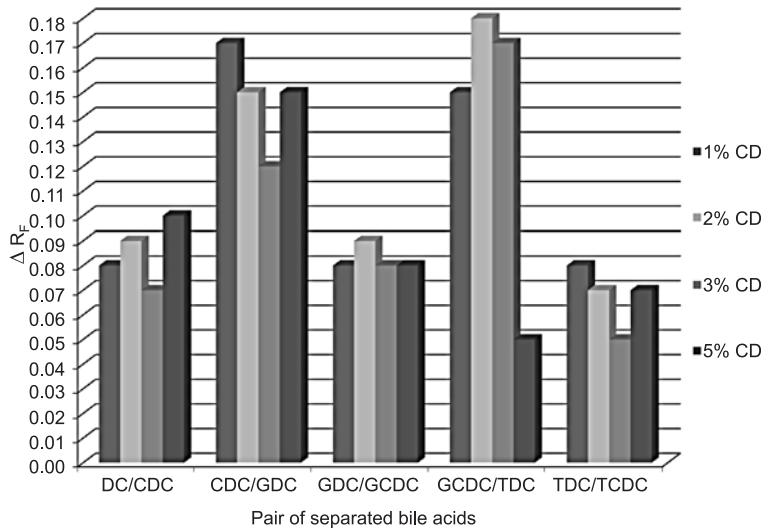


Figure 2. The effect of β-CD concentration as mobile phase additive on separation factor ($\Delta R_F$) of studied bile acids determined on RP-2$F_{254}$ plates developed with the mobile phase methanol-water-β-CD in volume composition 40 : 10 : 5. The concentration of β-CD was: 1, 2, 3 and 5%, respectively

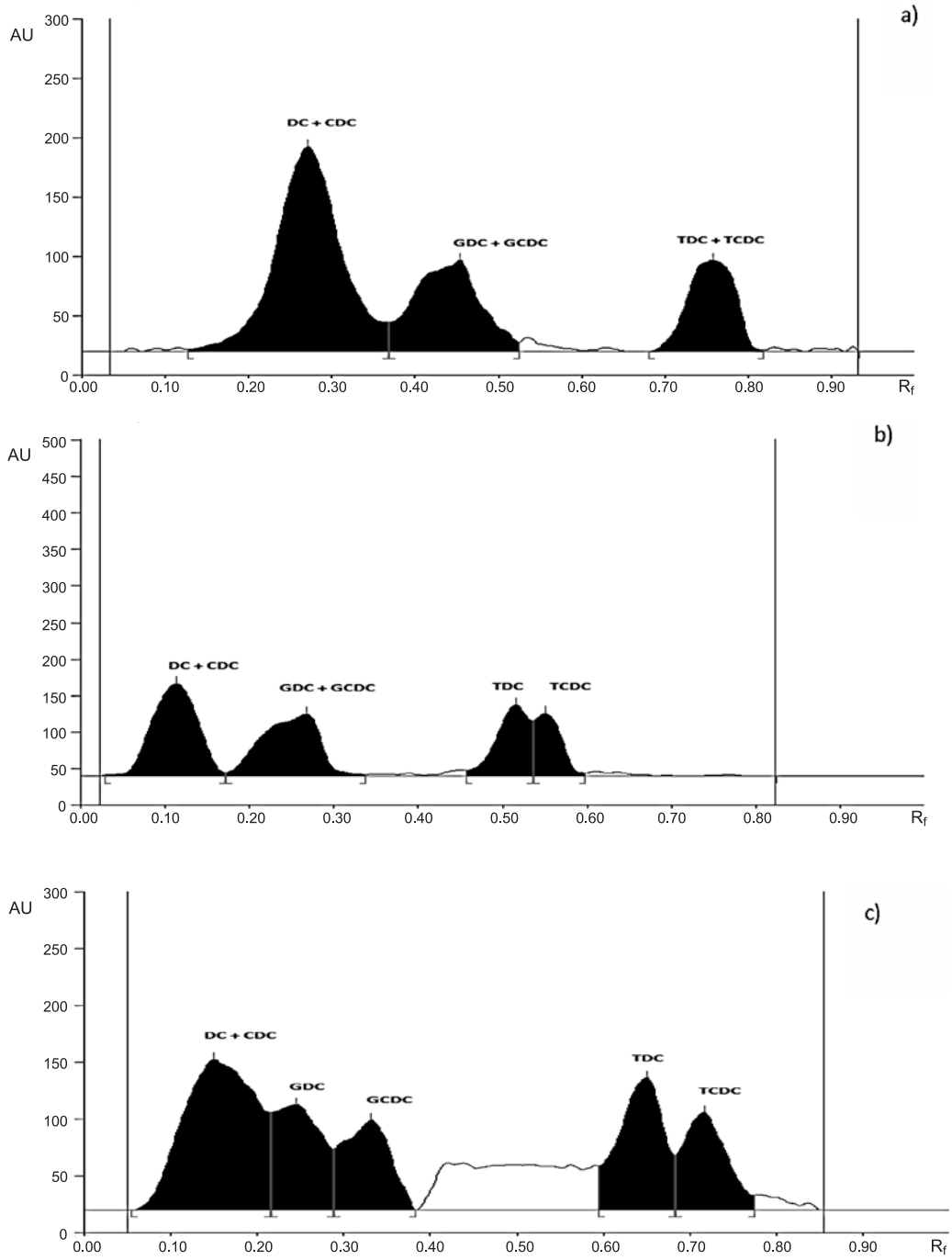


Figure 3. Chromatograms showing the resolution of examined bile acids on reversed phase plates RP-8$F_{254}$ developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-β-CD (0.5%) in volume composition 40 : 10 : 5 (b), methanol-water-β-CD (2%) in volume composition 40 : 10 : 5 (c)

examined bile acids significantly. The chromatograms of mixture consisted of DC, CDC, TDC, TCDC, GDC and GCDC acids separated on RP-2$F_{254}$ plates with the use of this mobile phase, where β-CD concentration was: 0.5, 1, 3%, and also to compare those produced by mobile phase without β-CD are presented in Figure 1. Good separated peaks coming from investigated mixture of bile acids show the

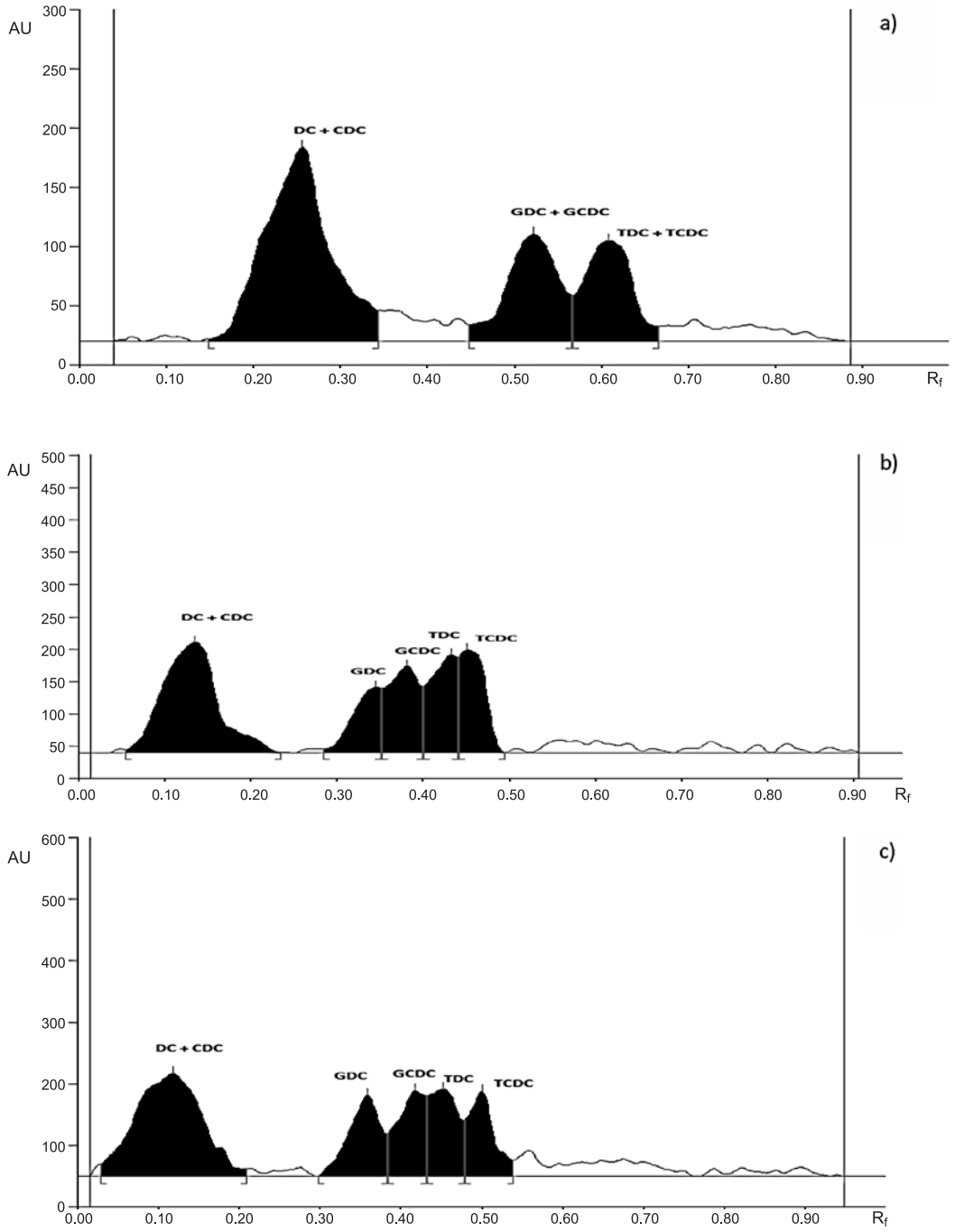


Figure 4. Chromatograms showing the resolution of examined bile acids on reversed phase plates RP-18$F_{254}$ developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-β-CD (0.5%) in volume composition 40 : 10 : 5 (b), methanol-water-β-CD (2%) in volume composition 40 : 10 : 5 (c)

exemplary chromatograms presented in Figure 1 c and d. On the basis of Figure 1, it could be observed that addition of β-CD at concentration from 1 to 5% to the mobile phase enabled resolution of all investigated compounds. The best results of separation of studied bile acids, especially DC and CDC can be observed on RP-2$F_{254}$ plates developed using mobile phase containing 3% β-CD (Fig. 1 d). In this case,

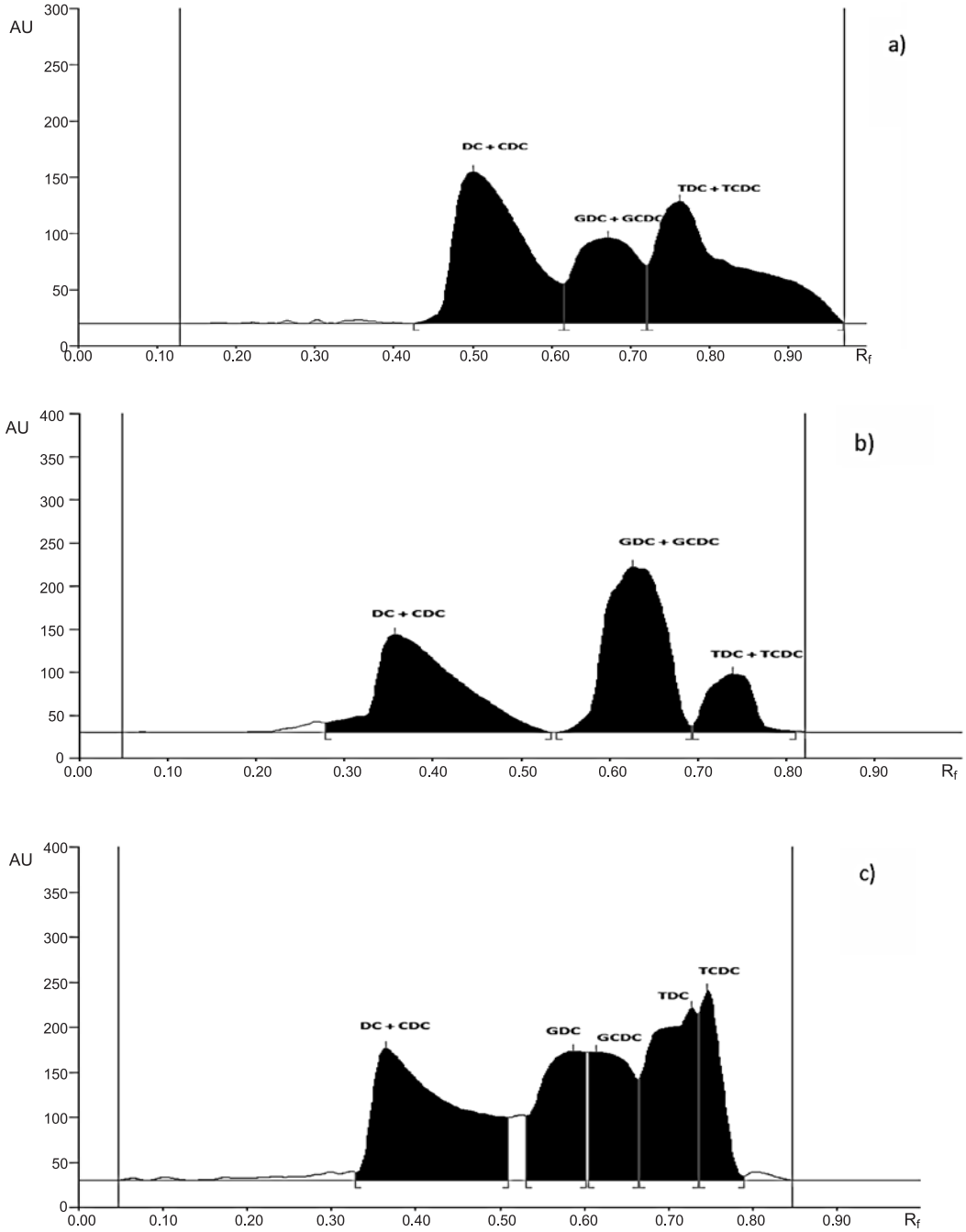


Figure 5. Chromatograms showing the resolution of examined bile acids on HPTLC chiral plates developed with the following mobile phases: methanol-water (40 : 10, v/v) (a), methanol-water-β-CD (1%) in volume composition 40 : 10 : 5 (b), methanol-water-β-CD (2%) in volume composition 40 : 10 : 5 (c)

there are two separated peaks coming from both bile acids with regular shape. Moreover, to confirm this complete separation, comparison the values of separation factor ($\Delta R_F$) calculated for each pair of examined bile acids, which have been determined on the basis of chromatograms produced using 1, 2, 3, and 5% solutions of β-CD and RP-$2F_{254}$ plates was done (Fig. 2). Complete resolution of each pair (two

neighboring bile acids) enables $\Delta R_F$ = 0.05. The results of $\Delta R_F$ calculated for five pairs of studied bile acids show that the proposed chromatographic conditions allowed complete resolution of all compounds because the $\Delta R_F$ values obtained for five pairs of examined bile acids: DC/CDC, CDC/GDC, GDC/GCDC, GCDC/TDC and TDC/TCDC are higher than 0.05 in each case. Figure 2 confirms that applied β-CD as mobile phase additive improves the resolution of free bile acids: DC and CDC and their conjugates, especially CDC/GDC and also GCDC/TDC.

Next applied reversed phase plates were RP-$8F_{254}$. The exemplary chromatograms obtained by means of this stationary phase and mobile phases: methanol-water without adding β-CD and also these containing 0.5% and 2% aqueous solution of β-CD confirm that β-cyclodextrin improves separation of examined bile acids, in particular glycine and taurine conjugates of DC and CDC, such as GDC/GCDC and TDC/TCDC (Fig. 3 a, b, c). Unfortunately, none of applied modification of mobile phase by use of β-CD at concentration: 0.5, 1, 2, 3 and 5% did not allow for successful separation of all examined pairs of six bile acids. The biggest problem in the case of discussed silica gel RP-$8F_{254}$ was to achieve complete separation of DC from CDC.

Continuing this research on bile acids separation by 1D RP-TLC system and β-CD as mobile phase component, next type of commercially available reversed phase plates RP-$18F_{254}$ was applied. Chromatograms of separated bile acids obtained on RP-$18F_{254}$ plates developed using mobile phase containing β-CD at concentration 0.5, 1, 2, 3 and 5% indicate that addition of β-CD to applied mobile phase was suitable to improve the separation of glycine and taurine conjugates of examined bile acids, such as GDC from GCDC and also TDC from TCDC, but it did not allow to solve the problem of separation of difficult to separate pair of free bile acids DC/CDC (Fig. 4 a, b, c). Thus, it could be concluded that RP-$18F_{254}$ plates allow to obtain very similar results of separation of investigated mixture like above described RP-$8F_{254}$ plates and these plates may be applied as an alternative to RP-$8F_{254}$ in bile acid separation.

In the third part of this study, the commercially available HPTLC chiral plates were applied. Addition of β-CD at concentration from 0.5 to 5% to the mobile phase: methanol-water (40 : 10, v/v) in quantity of 5 mL improved the resolution of the following bile acids: GDC/GCDC and TDC/TCDC but the effect of separation of free DC and CDC was not so satisfactory like in the case of previously presented RP systems (Fig. 5 a, b and c). Applied chromatographic conditions did not enable separation of DC and CDC. Thus, it could be suggested that of two 1 D TLC systems tested in this work, RP is more efficient than NP for the separation of optically active bile acids, such as DC and CDC and their conjugated forms. Developed 1D RP-HPTLC system with the use of RP-$2F_{254}$ plates and methanol-water-β-CD (40 : 10 : 5, v/v/v) as mobile phase is the most suitable for the separation and characterizing of examined bile acids, including unconjugated forms of DC and CDC. Other applied chromatographic plates (RP-$8F_{254}$, RP-$18F_{254}$ and also HPTLC chiral plates) and described mobile phase can improve only the separation of glycine and taurine conjugates of studied bile acids: GDC, GCDC, TDC and TCDC.

## CONCLUSIONS

In conclusion, the authors of presented TLC-densitometric study confirmed that described components of both applied TLC systems, like mobile phase content (especially β-CD concentration) and also the type of chromatographic plates can improve the resolution of proper pair of free and also conjugated with glycine and taurine deoxycholic and chenodeoxycholic acids. Of all applied chromatographic conditions, RP-$2F_{254}$ plates and one dimensional (1D) system with the use of mobile phase: methanol-water-β-cyclodextrin in volume composition 40 : 10 : 5 at different concentration of aqueous solution of β-cyclodextrin (1, 2, 3, 5%) are useful to complete resolution and characterizing of mixture containing all examined bile acids including difficult to separate free deoxycholic and chenodeoxycholic acids. The conjugated with taurine (TDC, TCDC) and with glycine (GDC, GCDC) bile acids can be satisfactory separated also on other chromatographic plates described in this paper like, for example, RP-$8F_{254}$ or RP-$18F_{254}$, respectively, developed by proposed mobile phase consisting of β-CD.

The developed RP-HPTLC procedure in one dimensional system is simple to use and rapid, thus, it can be used in preparative step of medical or pharmaceutical analysis of studied bile acids by modern chromatographic techniques, such as high-performance liquid chromatography or gas-liquid chromatography.

### Acknowledgment

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## DRUG BIOCHEMISTRY

# DESIPRAMINE, FLUOXETINE AND TRANYLCYPROMINE HAVE DIFFERENT EFFECTS ON APOPTOSIS INDUCED IN RAT CORTICAL NEURONS BY OXYGEN-GLUCOSE DEPRIVATION

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**Abstract:** Intensive *in vivo* and *in vitro* studies aimed to unravel new means of neuronal cell rescue from ischemic/hypoxic brain injury are in progress. Evaluation of the influence of a drug on cell viability and expression of apoptotic proteins in cell cultures exposed to oxygen-glucose deprivation (OGD) is used for assessment of their protective and anti-apoptotic properties. It is supposed that anti-apoptotic effects are involved in the therapeutic activity of antidepressants. The aim of the present study was to evaluate anti-apoptotic effect of desipramine, fluoxetine and tranylcypromine in OGD of cortical neurons. Cell cultures were exposed to OGD (3% of $O_2$) and one of the studied drugs at a concentration of 0.1, 1 or 10 μM for 6, 12 or 24 h. The drugs positively influenced cell viability estimated by MTT [3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl**t**etrazolium bromide] assay but their effect declined during incubation longer than 6 h. Only fluoxetine in all applied concentrations decreased the number of cells with the fragmented nuclei visualized by Hoechst staining after 24-h incubation. Moreover, fluoxetine stronger than desipramine and tranylcypromine stimulated Bcl-2 (**B**-**c**ell **l**ymphoma protein 2) expression evaluated by Western blotting in 6-h and 24-h experiments. Fluoxetine (0.1 or 1 μM) and tranylcypromine (0.1 μM) in time dependent manner induced the positive effect on pGSK3β(Ser9) (Ser 9 **p**hosphorylated **g**lycogen **s**ynthase **k**inase 3**β**) expression. Our results indicate that fluoxetine more efficiently than desipramine or tranylcypromine prevented OGD-induced apoptosis in the primary neuronal culture.

**Keywords:** antidepressant drugs, apoptosis, glycogen synthase kinase 3β, oxygen-glucose deprivation, primary neuronal culture

Several lines of evidence suggest that programmed cell death not only plays an important role in developmental processes of neural tissue but may also contribute to the loss of neurons observed in CNS structures of aging brain and in patients suffering from affective disorders as well as other CNS diseases, e.g., schizophrenia, neurodegenerative diseases, panic disorder. This process may underlie a common pathophysiological mechanism shared by several mental disease processes and provide some insight into the high rates of comorbidity that exist between neuropsychiatric disorders. It is assumed that the mechanism of action of numerous drugs modulating CNS function is associated with their protective effect on neurons and/or astrocytes (1-4). It is well known that process of apoptosis is characterized by chromatin condensation, DNA fragmentation and finally cell disintegration. The key players are cysteine proteases known as caspases which degrade proteins that are important for cell survival. Activity of these enzymes is regulated *via* the mechanism involving release of several apoptotic factors from mitochondria (e.g., apoptosis inducing factor, cytochrome C). It has been also evidenced that Bcl-2 family of proteins has a critical role in the regulation of cell survival. Bcl-2 proteins are members of the anti-apoptotic protein family, that increase mitochondrial outer membrane integrity and inhibit cytochrome C release from mitochondria thus preventing caspase 9 activation. They exert neuroprotective and neurotrophic effect and when present at a proper high concentration may prevent neuronal cell death (5, 6).

The hypothesis that antidepressants may affect factors involved in neuronal death and protection has been widely examined. Different mechanisms of such neuroprotective effect have been proposed (7-9). During the last decade, a growing interest of sci-

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entists has been focused on importance of canonic Wnt/β-catenine pathway in CNS pathology. Nowadays, it is known that protein components of this pathway and kinases involved in the regulation of its activity play an important role in Alzheimer's disease, schizophrenia and bipolar affective disorders (10, 11). Glycogen synthase kinase 3 (GSK3) seems to be an interesting component of canonic Wnt (**W**ingless+i**nt**-1)/β-catenine signaling pathway. It has been found that phosphorylated GSK3β(Ser9) *via* β-catenine enhances transcription of several genes involved in processes of cell proliferation, differentiation, synaptic plasticity and apoptosis (12-14). Discovery of the lithium influence on GSK3 activity and its anti-depressant, mood improving and neuroprotective effects encouraged intensive studies on the participation of GSK3 in the mechanism of action of drugs affecting brain function (15, 16). The fact that GSK3 inhibition might regulate processes of neurogenesis and synaptic plasticity gave foundation to the idea that this kinase might be an important element of molecular mechanism of action of antidepressants (14, 17, 18). The observation that antidepressant drugs do not ameliorate disease symptoms immediately but usually after fourteen or more days from the onset of therapy may provide a rationale for such conviction. On the other hand, it is known that GSK3 also contributes to apoptosis (13). In fact, there are several data indicating that antidepressants target GSK3 signaling pathways and this ultimately promotes neurogenesis and their anti-apoptotic activity.

This study was designed to examine the effect of desipramine (a tricyclic antidepressant), fluoxetine (a representative of selective serotonin reuptake inhibitors) and tranylcypromine (a non-selective irreversible inhibitor of monoamine oxidase) on cell viability, apoptosis (Hoechst staining and Bcl-2 expression) and the level of pGSK3β(Ser9) in primary culture of cortical neurons subjected to combined oxygen-glucose deprivation. Expression/activity of both studied proteins - Bcl-2 and pGSK3 β(Ser9) is regulated *via* the same pro-survival PI3K/Akt (**p**hosphatidylinositol **3 k**inase/Protein kinase B) pathway.

Combined oxygen-glucose deprivation (OGD) is widely used as an *in vitro* model of ischemic/hypoxic insult. Parallel withdrawal of glucose and trophic factors from culture medium and decreased oxygen concentration induces alterations in cultured cell viability, development of apoptosis and disturbances of different intracellular signaling pathways. This model is considered to be useful for assessment of cytoprotective/anti-apoptotic potency of different drugs or chemicals (19) and that is why it was used in current study. Another rationale for testing antidepressant drug in this model is also fact that depression might develop in the aftermath of ischemic stroke episodes in some patients (20). To the best of our knowledge, there are only a few studies on the effect of antidepressants on OGD-induced apoptosis in primary cortical neuronal cultures and even when this model was applied, the studies were focused on reoxygenation events (21, 22). The aim of this study was to examine what is the influence of the chosen antidepressants on some markers of apoptosis in neurons exposed to OGD insult.

## MATERIALS AND METHODS

### Cell culture

Primary cultures of cortical neurons were prepared from Wistar rat embryos at day 18$^{th}$ of pregnancy according to the method of Toborek et al. (23). After dissection, brain tissue was first mechanically disrupted and then digested with a medium containing 1 mL of 10 × concentrated 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Sigma, USA) and 9 mL of DMEM (**D**ulbecco's **m**odified **E**agles **m**edium) (Invitrogen, USA). After a short incubation at 37$^{o}$C, the enzyme was inactivated with an equal volume of 10% FBS (**f**etal **b**ovine **s**erum) (Invitrogen, USA) containing DMEM (DMEM-FBS10) and centrifuged for 5 min at 1000 rpm (225 × g). Supernatant was discarded and cells were next dissociated using DNA-se I (Roche, Switzerland) solution (40 μg/mL of DMEM-FBS10). The cortical neurons were plated at a density of 2 million. cells/1 mL of DMEM-FBS10 into 96 well plates for viability measurement or on 60 mm polyethyleneimine (Fluka, USA) coated culture dishes (p60) (Nunc, Denmark or Becton Dickinson, USA) for other experiments (100 μL/well and 2 mL of cell suspension/dish, respectively). On the next day after seeding, culture medium was changed to Neurobasal medium with B27 supplement, L-glutamine (2 mM), gentamicin (100 μg/mL) and fungizone (2.5 μg/mL) (all from Invitrogen, USA). The medium was then exchanged every third day. Neuronal cultures were maintained in an atmosphere of 5% $CO_2$ and 95% relative humidity at 37$^{o}$C (NUAIR $CO_2$ Incubator, USA). To prevent growth of glial cells, fluordeoxyuridine (54 μM) and uridine (14 μM) mixture (Sigma, USA) was added to medium for 24 h. Some cultures were incubated with neuron-specific anti-MAP2 (**m**icrotubul **a**ssociated **p**rotein **2**) antibody or glia-specific anti-GFAP (**g**lial **f**ibrillary **a**cidic **p**rotein) antibody

(Sigma, USA) and stained with secondary Texas Red or FITC (fluorescein isothiocyanate) conjugated antibodies (Santa Cruz Biotech., USA) to confirm purity of cell cultures. Above 90% of cell population were MAP2 positive. Experiments were performed on $9^{th}$ – $12^{th}$ day after plating.

**Treatment of neuronal cultures**

Neuronal cell culture standard medium (Neurobasal) was replaced with glucose free DMEM containing examined antidepressant drugs and incubated for 6, 12 or 24 h in the ischemia simulating conditions (New Brunswick Scientific $CO_2$ Incubator Galaxy 48R, USA). The OGD model is based on restricted oxygen concentration (3% $O_2$, 92% $N_2$, 5% $CO_2$) and glucose and growth supplement (B27) withdrawal from culture medium containing mannitol instead to maintain adequate medium osmolarity (319 mOsm) (24). Cells were treated with the following antidepressants: desipramine [3-(10,11-dihydro-5*H*-dibenzo[*b*,*f*]-azepin-5-yl)-*N*-methylpropan-1-amine hydrochloride] (Sigma, USA), fluoxetine [(*RS*)-*N*-methyl-3-phenyl-3-[4-(triflurомethyl)phenoxy] propan-1-amine hydrochloride] (Polfa, Poland) and tranylcypromine [(±)-*trans*-2-phenylcyclopropan-1-amine] (Sigma, USA) at a concentration of 0.1, 1 and 10 µM for 6, 12 or 24 h. The concentrations of antidepressant drugs in this study were inside the clinically useful range. The brain concentration of antidepressant drugs vary from 5 to 25 µM after systemic administration of pharmacologically effective doses to animals and humans (25-27). Control neuronal cultures where maintained in normoxic condition or OGD condition only and not exposed to the antidepressant drugs.

The study was approved by the local ethics committee for animal experiments of the Medical University of Silesia.

**Cell viability**

To estimate general cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was performed. The cells ability to convert MTT indicates mitochondrial integrity and activity, which in turn reflects cell viability. MTT (Sigma, USA) dissolved in PBS (phosphate buffered saline) was added to the culture medium 3 h before the end of experiment at a final concentration of 0.25 mg/mL. Medium was then removed and cells were lysed with MSO (methyl sulfoxide) (Sigma, USA) to allow formazan crystals to dissolve. Absorbance at the wavelength of 590 nm was read on a Multiscan RC microplate reader (Labsystems, Finland). Influence of antidepressant drugs on neuronal cultures was first tested under normoxic conditions. Results are presented as the means absorbance + SD of three independent experiments.

**Assay for nuclear apoptosis**

Hoechst dye 33342 (Sigma, USA) was used to stain nuclei of cortical neurons. After 24 h of incubation at experimental conditions, cell cultures growing on 60 mm dishes were rinsed with PBS and fixed in methanol for 15 min at RT (room temperature), washed again with PBS and incubated with Hoechst dye (1 µg/mL) for 20 min at RT in the dark. Analysis of cell nuclei was conducted under a fluorescence microscope (Nikon, Japan). Twenty-four hours exposure time was appointed to show explicit effect of antidepressant drugs on chromatin structure.

Apoptotic nuclei were identified as nuclei with chromatin fragmentation or condensation. The percent of apoptotic nuclei (apoptotic *versus* total number of nuclei) was counted at 40× magnification on at least six randomly selected fields, containing about 100 cells each. The results are expressed as the means + SD of three independent cell cultures.

**Preparation of cell lysates**

Cells were harvested and lysed in ice-cold lysis buffer pH = 7.4 containing 50 mM Tris [tris (hydroxymethyl)aminomethane], proteases and phosphatases inhibitors (aprotinin, leupeptin, pepstatin A, PMSF (**p**henyl**m**ethyl**s**ulfonyl **f**luoride) and sodium orthovanadate, 150 mM NaCl, 1 mM EDTA, Igepal and 1% SDS (**s**odium **d**odecyl **s**ulfate) (all reagents from Sigma, USA). Disrupted cells suspensions from 2 or 3 p60 dishes were combined together as one sample. Samples were then centrifuged (12 000 × g, 15 min, 4°C). Supernatants were collected and stored at –20°C until electrophoresis was performed. Protein concentration was determined using Bradford reagent (Sigma, USA).

**Western blotting**

Equal amounts of total protein (25-50 µg) were loaded into 10% SDS-polyacrylamide gel (SDS-PAGE) wells and electrophoresis was performed using 25 mM Tris-glycine-SDS buffer (pH = 8.3) at 100 V (all reagents from Sigma, USA). The resolved proteins were transferred from the gel onto nitrocellulose membrane (Bio-Rad Laboratories Ltd., UK) in 48 mM Tris-glycine-SDS methanol containing (20% v/v) buffer (pH = 8.3) at 100 V for 1 h.

Transfer quality was confirmed by staining of the membranes with Ponceau S (Sigma, USA). Membranes were then washed with TBST (Tris buffered saline – Tween 20) buffer (20 mM Tris, 0.5

M NaCl, 0.5% Tween 20) to remove the dye and incubated with 5% non-fat milk in TBST for 1 h at RT on orbital shaker to prevent non-specific binding of antibodies. The blots were incubated overnight at 4°C with specific primary antibodies (concentration range 1 : 200 – 1 : 1000) raised against a protein of interest, e.g., Bcl-2 anti-apoptotic protein, glycogen synthase kinase 3β (GSK3β) and phosphorylated pGSK3β (Ser9) form (inactive) (all antibodies from Santa Cruz Biotech., USA).

On the next day, the membranes were rinsed 3 times (10 min each) in a copious amount of TBST and incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at RT (1 : 2000 in 5% milk/TBST solution). Membranes were rinsed in TBST as described previously and briefly (1 min) incubated with ECL (**e**nhanced **c**hemi**l**uminescence) system reagents (GE Health Care, UK). Results were detected by exposing Kodak XAR-5 film (Sigma, USA) to luminescent signal. Protein bands intensity was assessed by densitometry measurement (Image J Software 1.42 q, NIH, USA). Results were expressed as percent of OGD control and reported as the means + SEM of 3 independent cell cultures.

### Statistical analysis of results

Statistical analysis of the data was performed using a one-way ANOVA followed by *post-hoc* Newman-Keuls test (GraphPad Prism 4.01 software Inc., USA). Statistical probability above 95% ($p < 0.05$) was considered significant.

## RESULTS

### Cell viability

Neuronal cell viability was not affected by the antidepressant drugs in the applied range of concentrations under normoxic conditions (data not shown). OGD exposure for 6, 12 or 24 h diminished neuronal viability measured by MTT assay by 33, 45 or 57%, respectively. During a 6-h exposure to OGD and the

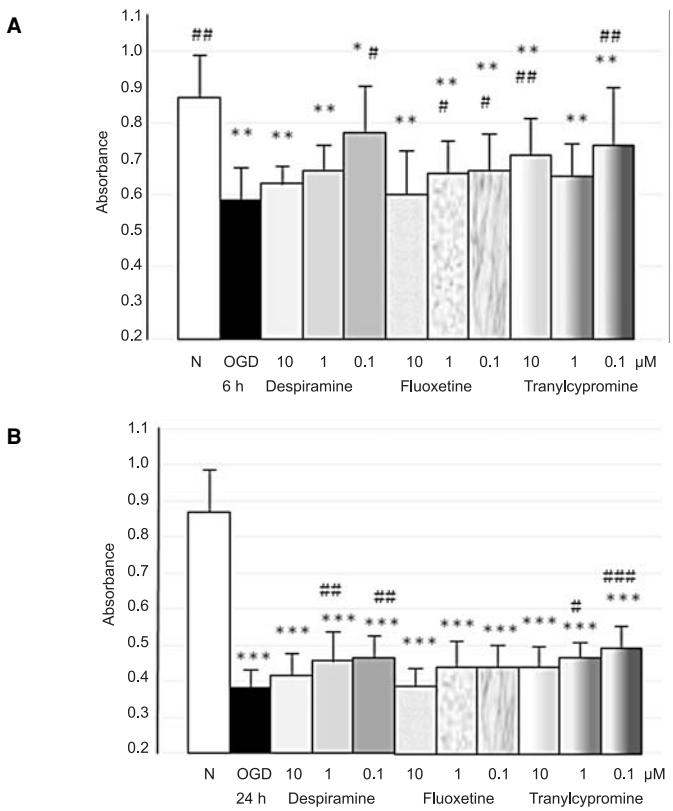


Figure 1. Cell viability measured by MTT reduction assay. The results are expressed as the mean (n = 72) absorbance measured + SD (data collected from 3 independent experiments). Desipramine, fluoxetine and tranylcypromine were applied at a concentration of 10, 1 and 0.1 µM. (A) 6-hour experiment (B) 24-hour experiment. N = normoxia condition, standard culture media; OGD = oxygen-glucose deprivation condition. Newman-Keuls procedure after one-way ANOVA was applied. Statistical significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* normoxia; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs.* OGD

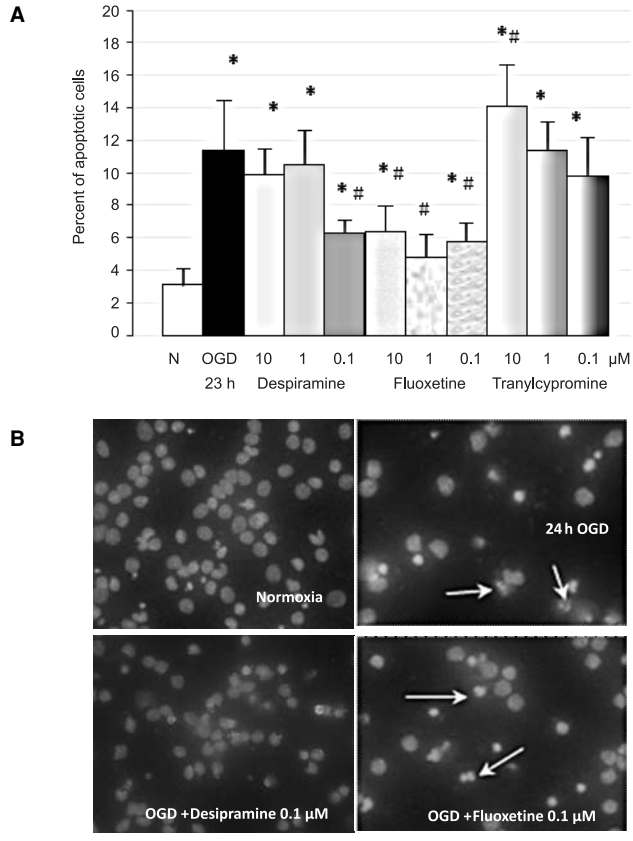


Figure 2. Hoechst staining. (A) The quantity of apoptotic cells (percent of total cell number) after 24 h exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 µM. The results are expressed as the mean (n = 18) + SD. N = normoxia condition, standard culture media; OGD = oxygen-glucose deprivation condition. Statistical significance (Neuman-Keuls test after one-way ANOVA): * $p < 0.001$ (*vs*. normoxia), # $p < 0.001$ (*vs*. OGD). (B) Pictures from under the fluorescent microscope, magnification 600 ×

studied antidepressants, cell viability was increased in comparison to the OGD effect alone. This effect was induced by desipramine, fluoxetine and tranylcypromine at the lowest concentration of 0.1 µM (an increase by 32, 15 and 26%, respectively) as well as by fluoxetine at the concentration of 1 µM (by 13%) and by tranylcypromine at the highest concentration of 10 µM (by 22%) (Fig. 1A). After a 12-h exposure to OGD and desipramine or fluoxetine, neuronal viability was not changed in comparison to OGD alone but was increased after incubation with tranylcypromine at the concentration of 0.1 or 1 µM (about 14%) (data not shown). In the 24-h experiment, desipramine and tranylcypromine at both lower concentrations of 0.1 and 1 µM increased cell viability: desipramine about 17% and tranylcypromine by 17 or 23%, respectively. Also fluoxetine at these concentrations induced a tendency towards an increased cell viability (Fig. 1B).

**Hoechst dye staining**

In order to estimate the influence of the antidepressants studied on intensity of chromatin condensation and nuclei disintegration induced by OGD, cell cultures were exposed to OGD and antidepressants for 24 h. In OGD-exposed cultures, the apoptotic cell population increased markedly up to 11% in comparison to about 3% of such cells visible under normoxic conditions (Fig. 2B). In comparison to the effect of OGD alone, desipramine at the concentration of 0.1 µM and fluoxetine in all applied concentrations reduced the number of apoptotic cells by about 50%. Unexpectedly, tranylcypromine significantly enhanced DNA fragmentation at the concentration of 10 µM up to 14% but a tendency towards a reduced number of cells with apoptotic nuclei was observed when the drug was used at the lowest concentrations (Fig. 2A).

**Expression of Bcl-2 protein**

Under OGD conditions, a tendency towards an increased expression of Bcl-2 protein was noted in neuronal cultures exposed for 6 h to desipramine or fluoxetine. Only fluoxetine at a concentration of 0.1 μM produced significant effect - an increase by 18% (Fig. 3A).

The studied drugs did not affect Bcl-2 protein levels after 12 h of incubation (data not shown). The more pronounced positive tendency was observed after 24-h incubation with desipramine as well as fluoxetine in all concentrations and tranylcypromine at concentration of 0.1 μM. The significant increase by 32% was induced only by fluoxetine at the lowest concentration (Fig. 3B).

**Expression of pGSK3β(Ser9) and pGSK3β(Ser9)/GSK3β ratio**

In the result of a 12-h incubation under OGD conditions, desipramine did not affect the expression of pGSK3β(Ser9) with the exception of 1 μM concentration. Effect was not observed after standardization to GSK3β. The relative blot intensity was increased by 10% in comparison to its value estimated after a 6-h incubation with desipramine. Fluoxetine in a concentration-dependent manner, namely at 0.1 μM (by 27%), 1 μM (by 20%) and at 10 μM (by 15%) increased the expression of pGSK3β(Ser9). Similar effect was observed in the pGSK3β(Ser9)/GSK3β ratio. Tranylcypromine increased the expression of pGSK3β(Ser9) and a relative blot intensity only at a concentration of 0.1 μM (Fig. 4B and 5B).

In 24-h experiment, only tranylcypromine when applied at 0.1 μM decreased pGSK3β(Ser9) expression apparently but had no influence on the relative blot intensity (Fig. 4C and 5C).

**DISCUSSION**

The present study was performed on primary cortical neuronal cultures in the well-known oxy-

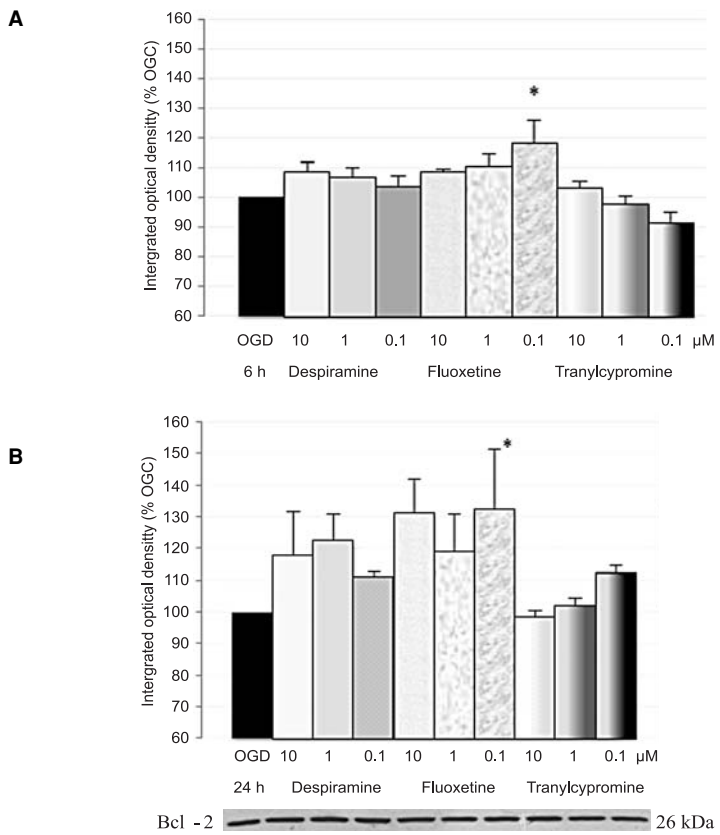


Figure 3. Bcl-2 protein expression after exposure to OGD and desipramine, fluoxetine or tranylcypromine treatment at a concentration of 10, 1 and 0.1 μM. The results are expressed as the mean (n = 4) percent of blot integrated optical density measured in OGD only exposed cell cultures + SEM. OGD = oxygen-glucose deprivation condition. (A) 6-h experiment (B) 24-h experiment. Statistical significance (Neuman-Keuls test after one-way ANOVA): * $p < 0.05$ *vs*. OGD. Representative blots of Bcl-2 (upper blot) and β-actin (as a protein load control) are attached

gen-glucose deprivation model. To the best of our knowledge, the effect of antidepressants on OGD-induced apoptosis was mainly evaluated during re-oxygenation period which is considered as a phase more responsible for the ischemic toxic effects (7, 28, 29) but not immediately after OGD neurotoxic insult as it was in the present study.

As demonstrated by Hoechst staining, the most pronounced anti-apoptotic effect in cells exposed to OGD for 24 h was induced by fluoxetine at all applied concentrations. Desipramine to the same extent reduced the number of apoptotic cells only at the lowest applied concentration (0.1 μM). The results of the whole our study indicate that tranylcypromine is able to trigger or attenuate apoptosis in concentration-dependent manner. This drug induced the opposite effects when applied at the concentration of a 0.1 μM or at a 100-fold higher concentration - 10 μM. A positive tendency towards a decreased population of apoptotic cells we observed

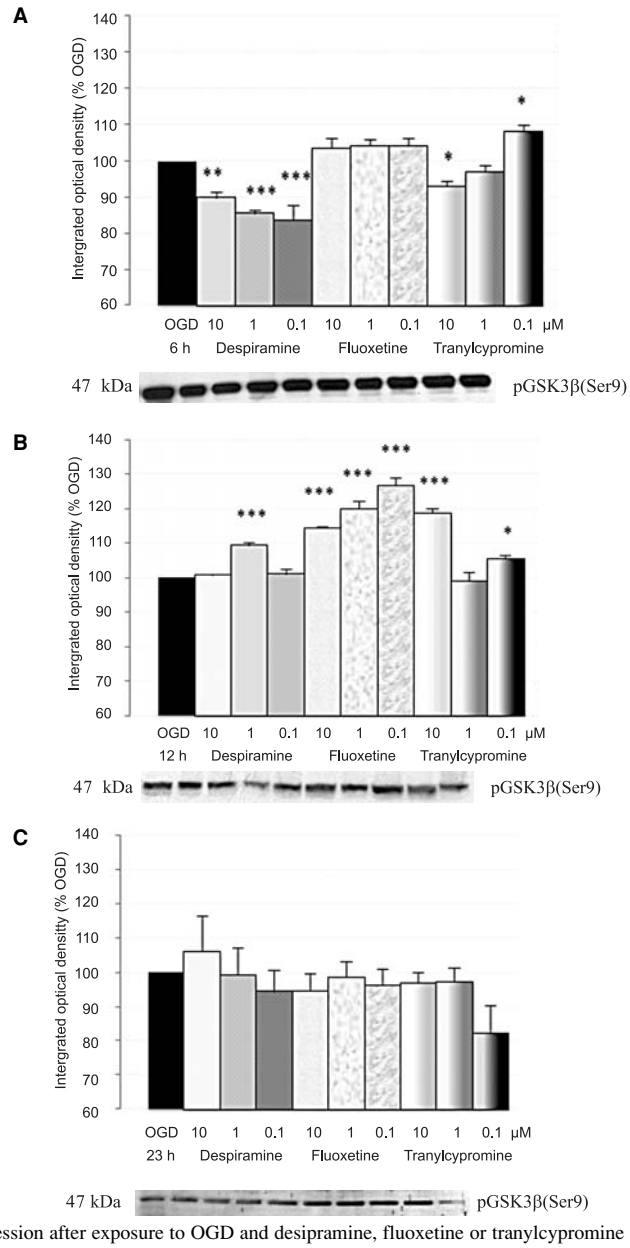


Figure 4. pGSK3β(Ser9) expression after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 μM. The results of pGSK3β(Ser9) level are expressed as the mean (n = 4) percent of blot integrated optical density measured in OGD only exposed cell cultures + SEM. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition. Statistical significance (Newman-Keuls test after one-way ANOVA): * $p < 0.001$; # $p < 0.01$ *vs.* OGD. Representative blots of pGSK3β(Ser9) are attached

in cultures exposed to OGD and the lowest concentration of tranylcypromine but at a concentration of 10 μM tranylcypromine even enhanced negative effect of OGD on nuclear DNA fragmentation. Similarly, Maruyama and Naoi (30) found the strongest neuroprotective effect of rasagiline (a selective irreversible MAO-B inhibitor) at a concentration of 0.1 μM on human neuroblastoma cell line. In the presented investigation, the studied drugs induced only a moderate positive effect on cell viability mainly observed after short, 6-h exposure to OGD. One may assume that discrepancy between the cell viability and Hoechst staining results is due to the reported by others inhibitory effect of fluoxetine and amitryptiline, for example, on mitochondrial respiratory chain enzymes (31).

In opposite, tranylcypromine was reported to have a positive influence on mitochondrial function

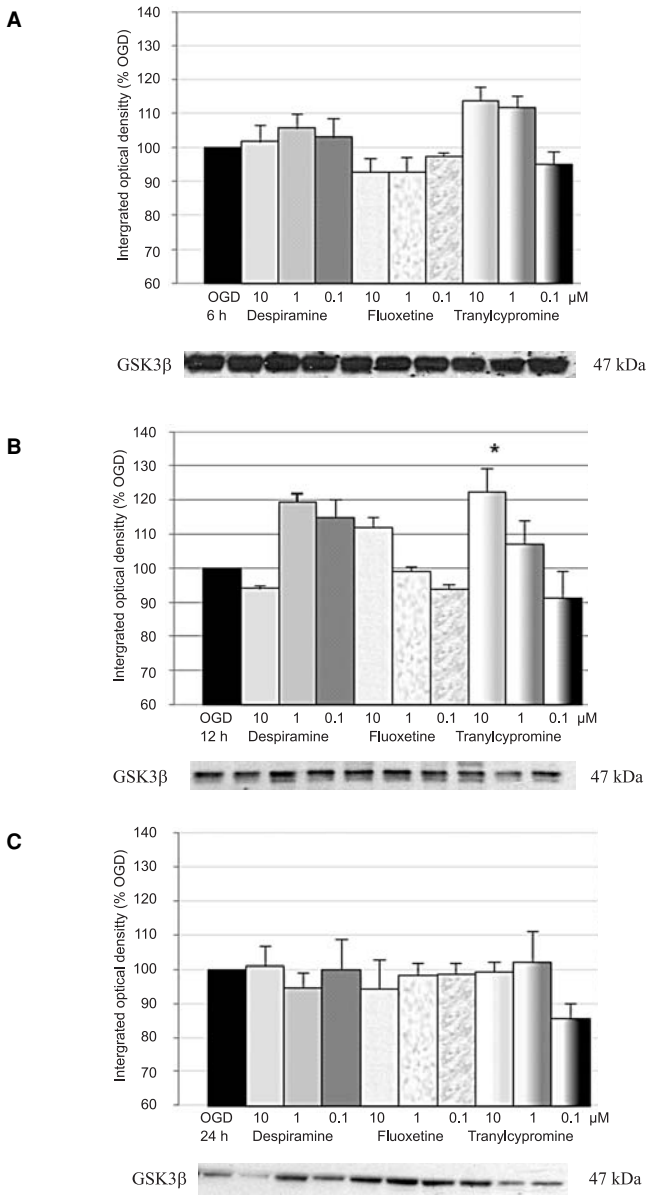


Figure 5. Total GSK3β expression after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 μM. The results of GSK3β level are expressed as the mean (n = 4) percent of blot integrated optical density measured in OGD only exposed cell cultures + SEM. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition. Statistical significance (Newman-Keuls test after one-way ANOVA): * $p < 0.05$ *vs*. OGD. Representative blots of GSK3β are attached

and in our study, a positive tendency towards an increased cell viability in cultures exposed to tranylcypromine at the lower concentrations was also observed (32). The effects of the studied drugs on mitochondria seems to be important because MTT assay used to evaluate cell viability is an indicator of mitochondrial function.

Moreover, our findings indicate that especially fluoxetine has positive influence on expression of Bcl-2. The observed up-regulation of Bcl-2 expression was parallel to anti-apoptotic effects (detected by Hoechst staining) of desipramine, fluoxetine and tranylcypromine at the lowest applied concentration. The obtained results are in line with the data from the other studies in which the anti-apoptotic effects of fluoxetine and desipramine were observed in studies conducted *in vitro* or in a few *in vivo* experiments (25, 33). Tranylcypromine was not as intensively studied as other MAO inhibitors in regard to its neuroprotective potential. Kosten et al. (6) reported an increased expression of the Bcl-2 and Bcl-XL (**B**-**c**ell **l**ymphoma-e**x**tra **l**arge) mRNA measured in

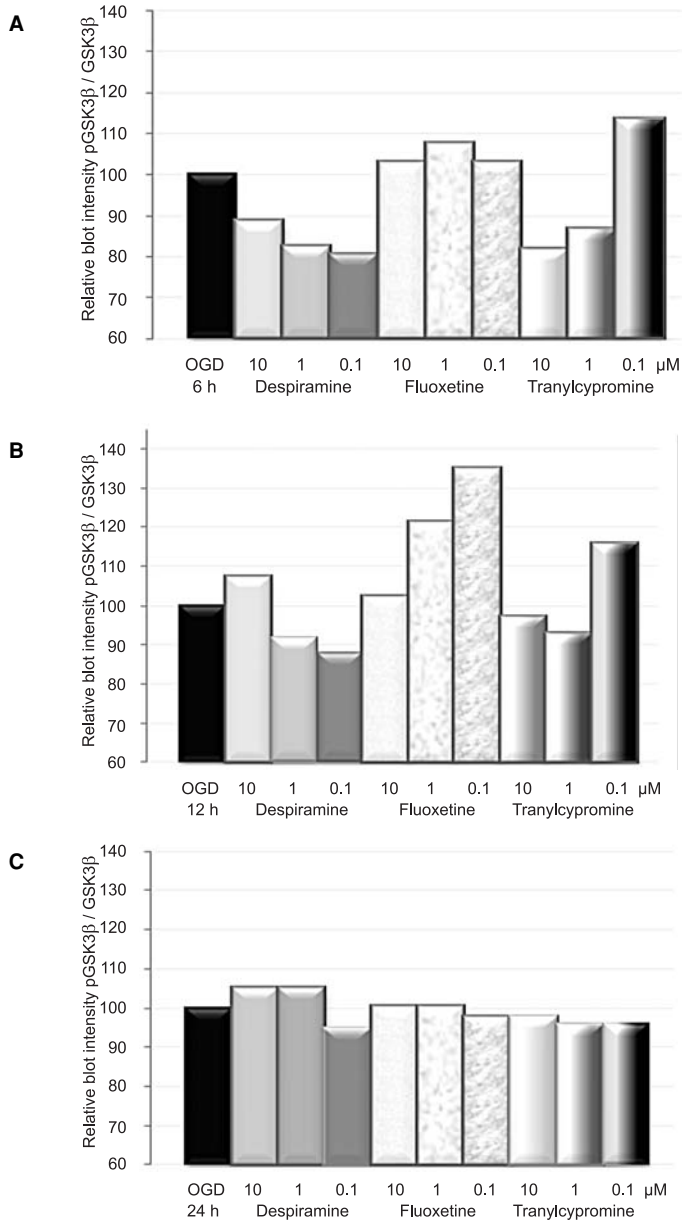


Figure 6. Relative blot intensity of pGSK3β(Ser9)/total GSK3β after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 μM. The results are expressed as the percent of relative optical density calculated for OGD only exposed cell cultures. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition

different rat brain structures after tranylcypromine administration. In our study, the effect of tranylcypromine on Bcl-2 expression was time and concentration dependent. Parallel positive influence of tranylcypromine on cell viability and on Bcl-2 expression was found only in 24 h experiment.

Futhermore, the results obtained in this study reveal that the examined antidepressants display different impact on GSK3β phosphorylation. GSK3β plays a role as a pro-apoptotic agent. Activation of Wnt signaling increases GSK3 inactivation by serine phosphorylation in position 9 of the GSK3β isoform or position 21 of GSK3a, respectively. Hence, factors intensifying phosphorylation of GSK3β can prevent initiation of programmed cell death. Numerous papers concerning mood stabilizers or effects of SSRI have suggested that GSK3β inhibition produces both anti-apoptotic and neuroprotective effects through the influence on neurogenesis and synaptic plasticity and/or remodeling (14, 17, 18, 34).

In the applied OGD model, fluoxetine induced a more beneficial influence, namely, it preserved or increased pGSK3β(Ser9) level. These results supported the mentioned above anti-apoptotic effect of fluoxetine. However, we did not observe the parallel effect of desipramine on pGSK3β expression and percentage of apoptotic cells induced by OGD. Influence of tranylcypromine on GSK3β phosphorylation was not homogenous. Also in this experiment, the strongest positive effect was found when the drug was applied at the concentration of 0.1 μM for 6 or 12 h.

Until now, only several studies in the primary neuronal cultures or in the *in vitro* OGD model have been performed to investigate the influence of desipramine or fluoxetine used in the present study on GSK3β activity (28, 35).

Obtained results suggest that desipramine and tranylcypromine especially at concentration higher than 0.1 μM have an essential influence on some other mechanisms or signal transduction pathways but not Wnt/β-catenine signaling. To the best of our knowledge, the effect of neither desipramine nor tranylcypromine on OGD-induced apoptosis in the primary neuronal cultures has not been studied. Apart from PI3/Akt kinase and Wnt signaling pathways that regulate GSK3 activity, other mechanisms are considered to be involved in the anti-apoptotic drug effect in the OGD model. OGD-induced damage in PC12 (**p**heo**c**hromocytoma) cells, associated with a marked activation of ERK (**e**xtracellular signal **r**egulated **k**inase), JNK (c-**J**un **N**-terminal **k**inase) and p-38 MAPK (**m**itogen **a**ctivated **p**rotein **k**inase) was prevented by exogenous neuronal growth factor mediated JNK inhibition (36). Tranylcypromine has positive effect on MPTP (1- methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced neurotoxicity (32) or enhanced CREB (**c**AMP **r**esponsive **e**lement **b**inding transcription factor) mRNA level in hippocampus (37) - thus could possibly stimulate neurogenesis. There are some evidence indicating that antidepressants may enhance synthesis or release of neurotrophic factors from neurons. They presumably modify the signal transduction pathways associated with neurotrophic factor receptor stimulation. This may also explain their neuroprotective and/or anti-apoptotic effects (7, 12, 38).

To sum up, in the OGD experimental model, fluoxetine exerted stronger anti-apoptotic effects than desipramine or tranylcypromine in primary cortical neuronal culture under hypoxic insult as demonstrated by Hoechst staining, MTT cell viability test, Bcl-2 and pGSK3β(Ser9) expression level determination. It occurred that tranylcypromine is able to trigger or attenuate apoptosis in a concentration dependent manner. The obtained results suggest that the effect of the studied antidepressants on apoptosis induced by OGD *in vitro* only partly is mediated by GSK3β. Better recognition of neuroprotective activity of antidepressants is important because drugs with stronger such activity could be more preferable in the treatment of mood disturbances related especially to neurodegenerative diseases or in the therapy of post-stroke patients.

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# CONJUGATED LINOLEIC ACIDS (CLA) DECREASE THE BREAST CANCER RISK IN DMBA-TREATED RATS

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**Abstract:** The aim of this study was to investigate how supplementation of diet of female Sprague-Dawley rats with different doses of conjugated linoleic acids and for a varied period of time influences breast cancer risk, fatty acids profile and lipids peroxidation in chemically induced mammary tumors. Animals were divided into nine groups with different modifications of diet (vegetable oil, 1.0 or 2.0% of CLA) and period of supplementation, which lasted after (A), before (B) and before and after (BA) carcinogenic agent - 7,12-dimethylbenz[a]anthracene administration at 50th day of life. Mammary adenocarcinomas occurred in all groups, but CLA supplementation decreased the cancer morbidity. Two percent CLA seem to be excessive because of the coexisting cachexia. Two CLA isomers (cis-9, trans-11 and trans-10, cis-12) were detected in tumors but content of rumenic acid was higher. Dietary supplementation significantly influenced some unsaturated fatty acids content (C18:2 n-6 trans, C20:1, C20:5 n-3, C22:2), but the anti- or prooxidant properties of CLA were not confirmed. CLA can inhibit chemically induced mammary tumors development in female rats, but their cytotoxic action seems not to be connected with lipids peroxidation. CLA isomers differ with their incorporation into cancerous tissues and they influence the content of some other fatty acids.

**Keywords:** CLA, DMBA, mammary tumors, rats

**Abbreviations:** AOil = group of rats supplemented with vegetable oil after DMBA administration, A1% = group of rats supplemented with 1.0% of CLA after DMBA administration, A2% = group of rats supplemented with 2.0% of CLA after DMBA administration, BOil = group of rats supplemented with vegetable oil before DMBA administration, B1% = group of rats supplemented with 1.0% of CLA before DMBA administration, B2% = group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil = group of rats supplemented with vegetable oil before and after DMBA administration, BA1% = group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% = group of rats supplemented with 2.0% of CLA before and after DMBA administration, CLA = conjugated linoleic acids, DMBA = 7,12-dimethylbenz[a]anthracene, FA = fatty acids, FAME = fatty acids methyl esters, GC = gas chromatography, MDA = malonyldialdehyde, TBARS = thiobarbituric acid reactive substances, TEB = terminal end buds

Fatty acids profile of consumed fat determines both the composition and the functions of body lipids (1, 2). Moreover, the quality and the quantity of dietary fat is considered as one of the major factors influencing the risk of cancer. Conjugated linoleic acids (CLA), which are the positional and geometric isomers of linoleic acid (cis-9, cis-12 C18:2, n-6, LA) with two cis/trans unsaturated bonds separated by one single bond, are naturally present in milk and dairy products as well as in fat from ruminants. They are investigated since 1970' as a group of polyunsaturated fatty acids with numerous health-promoting properties (3, 4). CLA isomers can modify the risk of many diet-relating disorders, such as: obesity, atherosclerosis, cardiovascular disease, diabetes, osteoporosis and different types of cancer (5), but their action depends on the conformation of the isomer. Moreover, precise mechanisms of their action are still under investigation (6). Among many suggested ways of action, interactions with essential fatty acids in their metabolic pathways and anti- or prooxidant properties are very interesting.

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Table 1. Characteristics of experimental groups.

| Group | AOil | A1% | A2% | BOil | B1% | B2% | BAOil | BA1% | BA2% |
|---|---|---|---|---|---|---|---|---|---|
| Diet I (before DMBA) | Lab. H | Lab. H | Lab. H | Lab. H + oil Lab. H | Lab. H + 1.0% CLA | Lab. H + 2.0% CLA | Lab. H + oil | Lab. H + 1.0% CLA | Lab. H + 2.0% CLA |
| Diet II after DMBA) | Lab. H + oil Lab. H | Lab. H + 1.0% CLA | Lab. H + 2.0% CLA | Lab. H | Lab. H | Lab. H | Lab. H + oil Lab. H | Lab. H + 1.0% CLA | Lab. H + 2.0% CLA |
| Number of individuals | 8 | 9 | 9 | 13 | 15 | 10 | 14 | 17 | 9 |
| Number of individuals with mammary tumors | 7 | 6 | 3 | 13 | 11 | 10 | 13 | 15 | 1 |
| Mammary tumor incidence | 87.5% | 67.0% | 33.0% | 100% | 73.0% | 100% | 93.0% | 88.0% | 11.0% |
| Total number of tumors | 13 | 8 | 3 | 28 | 24 | 18 | 63 | 28 | 3 |
| Number of tumors per individual (range) | 0 – 3 | 0 – 2 | 0 – 1 | 1 – | 5 | 0 – 6 | 1 – 4 | 0 – 15 | 0 – 5 0 – 3 |
| Mean number of tumors per individual | 1.6 | 0.9 | 0.3 | 2.2 | 1.6 | 1.8 | 4.5 | 1.6 | 0.3 |
| Weight of single tumor range) [g] | 0.45 – 11.93 | 0.62 – 8.51 | 1.12 – 4.48 | 0.01 – 21.71 | 0.03 – 11.83 | 0.77 – 7.74 | 0.03 – 13.64 | 0.01 – 5.62 | 0.47 – 1.91 |
| Total weight of tumors in group [g] | 48.93 | 21.48 | 6.75 | 75.00 | 31.70 | 61.00 | 69.96 | 37.41 | 3.53 |
| Age of first tumor appearance [day of life] | 93 | 143 | 112 | 118 | 145 | 110 | 126 | 134 | 113 |

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration.

Many researchers emphasize that oxidative stress is connected with the etiology of many above-mentioned diseases. Lipids peroxidation, especially polyunsaturated fatty acids peroxidation, as a result of oxidative stress generates many biologically active compounds. They can increase the oxidative damage in cells as they can live relatively long and are highly reactive. Aldehydes, which are one of the groups of metabolites, interact with intracellular compounds, especially with proteins and nucleic acids, and can break the cell functions (7, 8). Estimation of the fatty acids metabolites generated during the lipids peroxidation facilitates the explanation of the mechanism of arising of pathological conditions, as well as the influence of fatty acids on them.

The aim of this study was to investigate how supplementation of diet of female Sprague-Dawley rats with different doses of conjugated linoleic acids and for a varied period of time influences the breast cancer risk. To find out the mechanism of CLA action we also assessed the fatty acids profile and concentration of lipids peroxidation products (thiobarbituric acid reactive substances – TBARS) in chemically induced mammary tumors.

## MATERIALS AND METHODS

### Animals

The guiding principles in the use and care of laboratory animals as well as the whole experiment were approved by The Local Ethical Committee on Animal Experiments. Female Sprague-Dawley rats (n = 104, age – 30 days) were purchased from Division of Experimental Animals, Department of General and Experimental Pathology (Medical University of Warsaw, Warszawa, Poland). They were kept in animal room at 21$^{o}$C, in a 12 h light : 12 h dark cycle and during the whole research they were fed *ad libitum* a standard laboratory diet Labofeed H (Fodder plant "Morawski", Żurawia 19, Kcynia, Poland) and water. Applied laboratory diet is composed of 22.0% protein, 4.0% fat, 30.0% starch, 5.0% fibre, 6.5% minerals. After the arrival and 1-week adaptation period, the animals were randomly divided into 9 groups with different dietary supplementation. The total characteristics of experimental groups is shown in Table 1. Moreover, at 50th day of life, all animals received intragastrically *via* gavage a single dose of carcinogenic agent - DMBA (7,12-dimethylbenz[a]anthracene, approx. 95%, Sigma-Aldrich) in the amount of 80 mg/kg body weight. Diet of three groups was supplemented with conjugated linoleic acid (Bio-C.L.A., Pharma Nord, Denmark), given *via* gavage in the amount of 0.15 mL/day (1% in diet) and diet of three other groups was supplemented with conjugated linoleic acid (Bio-C.L.A., Pharma Nord, Denmark), given *via* gavage in the amount of 0.30 mL/day (2% in diet). Bio-C.L.A. contained two main CLA isomers: cis-9, trans-11 (31.4 ± 0.0%) and trans-10, cis-12 (33.3 ± 0.1%) as main fatty acids, and also oleic acid (11.0 ± 0.0%), linoleic acid (10.2 ± 0.0%), palmitic acid (4.6 ± 0.0%) and stearic acid (2.2 ± 0.0%). Three control groups received intragastrically vegetable oil in the amount of 0.15 mL/day. Applied oil does not contain conjugated linoleic acids and was purchased from Pharma Nord, Denmark, where it is used as the substrate to Bio-C.L.A. synthesis. This allowed us to minimize the differences in fatty acids profile of applied diets. Table 2 shows the fatty acid composition of applied diets. Supplementation of diet was conducted after (AOil, A1%, A2%), before (BOil, B1%, B2%) and before and after (BAOil, BA1%, BA2%) DMBA administration. During the experiment, rats were weighed weekly and palpated to detect the appearance of tumors. The entire experiment lasted for the following 21 weeks. In the 21st week of the experiment, most of the animals were decapitated and exsanguinated, and the weight of internal organs were determined. Only animals of A2% and BA2% groups were decapitated in the 15th week of the experiment, because of their much lower body weight and cachexia.

### Histopathological examination

DMBA treatment caused the mammary tumors induction, which effectiveness was determined as the percentage of animals with tumors in each group. Some of the tumors collected during necropsy (usually three in each group), selected randomly, were fixed in 10% formalin and were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland.

### Preparation of experimental material

Mammary tumors were collected during necropsy and stored at -20$^{o}$C until being analyzed.

### Fatty acids analysis

Fatty acid analysis was made by means of gas chromatography (GC) with capillary column and flame-ionization detection. Mammary tumors were thawed only once and three parallel samples of 0.2 g were taken for lipids extraction according to Folch et al. with minor modification (9). Purified organic extract was evaporated to dryness under a stream of

Table 2. Fatty acids composition of applied diets.

| Fatty acid [%] | Fodder + oil | Fodder + 1.0% CLA | Fodder + 2.0% CLA |
|---|---|---|---|
| C6:0 | 0.11 ± 0.01 | 0.12 ± 0.00 – | |
| C8:0 | – | 0.02 ± 0.00 | 0.05 ± 0.03 |
| C10:0 | 0.01 ± 0.00 | 0.03 ± 0.00 | 0.04 ± 0.00 |
| C11:0 | 0.01 ± 0.00 | – | 2.42 ± 0.01 |
| C12:0 | 0.02 ± 0.00 | 0.06 ± 0.08 | 0.02 ± 0.00 |
| C14:0 | 0.12 ± 0.01 | 0.12 ± 0.01 | 0.09 ± 0.05 |
| C15:0 | 0.07 ± 0.01 | – | 0.07 ± 0.00 |
| C16:0 | 10.49 ± 0.13 | 11.16 ± 0.23 | 10.12 ± 0.02 |
| C16:1 | 0.13 ± 0.00 | 0.13 ± 0.00 | 0.13 ± 0.00 |
| C17:0 | 0.08 ± 0.00 | 0.08 ± 0.00 | 0.07 ± 0.00 |
| C18:0 | 2.53 ± 0.01 | 2.47 ± 0.03 | 2.42 ± 0.00 |
| C18:1 n-9 | 34.46 ± 0.10 | 15.87 ± 0.16 | 15.59 ± 0.04 |
| C18:2 n-6 cis | 35.11 ± 0.05 | 31.46 ± 0.31 | 27.17 ± 0.07 |
| cis-9, trans-11 CLA | – | 8.57 ± 0.34 | 12.41 ± 0.13 |
| trans-10, cis-12 CLA | – | 8.47 ± 0.38 | 12.37 ± 0.10 |
| C18:3 n-3 | 14.15 ± 0.01 | 16.69 ± 0.19 | 13.70 ± 0.02 |
| C20:0 | 0.24 ± 0.12 | – | – |
| C20:1 | 0.36 ± 0.01 | 0.38 ± 0.00 | 0.49 ± 0.08 |
| C20:3 n-6 | 0.04 ± 0.06 | 0.01 ± 0.00 | – |
| C20:3 n-3 | 0.02 ± 0.00 | 0.04 ± 0.02 | – |
| C20:5 n-3 | 0.06 ± 0.01 | 0.10 ± 0.02 | 0.08 ± 0.00 |
| C21:0 | 0.06 ± 0.00 | 0.07 ± 0.00 | 0.06 ± 0.01 |
| C22:0 | 0.20 ± 0.10 | 0.16 ± 0.01 | 0.13 ± 0.01 |
| C22:1 n-9 | 0.04 ± 0.00 | 0.04 ± 0.00 | 0.03 ± 0.00 |
| C22:2 | 0.12 ± 0.00 | – | – |
| C23:0 | – | 0.12 ± 0.01 | – |
| C24:0 | 0.15 ± 0.04 | 0.06 ± 0.05 | 0.09 ± 0.05 |
| C24:1 | 0.10 ± 0.00 | – | – |

nitrogen and weighed to estimate the content of lipids in tumor tissue. The fat content was calculated as the percentage share of fat weight in tumor tissue [%]. The residue was taken for the preparation of fatty acids methyl esters (FAME) according to procedure of Bondia-Pons et al. with minor modifications (10), previously described for serum (11). Briefly, FAME were separated and quantified using Shimadzu GC-17A gas chromatograph with flame ionization detector. Injector was heated to 250°C and detector was heated to 270°C. Separation of FAME was performed on BPX70 capillary column (60 m × 0.25 mm i.d., film thickness: 0.20 µm, SGE) with helium as the carrier gas. The initial oven temperature was 140°C for 1 min, thereafter increased by 20°C/min to 200°C and hold for 20 min and then increased by 5°C/min to 220°C and hold for 25 min. Standards of CLA methyl esters: cis-9, trans-11 CLA and trans-10, cis-12 CLA were purchased from Nu-Chek-Prep. Inc., USA. Peaks of CLA isomers in examined samples were identified by comparison with retention time of standards and quantified by regression formula obtained with the standards. CLA content was expressed in relation to fat content and to tumor tissue weight.

**TBARS analysis**

From tumors thawed for fatty acids analysis, sample of 0.5 g was taken for TBARS analysis with spectrophotometric method (12). This sample was

mechanically homogenized with Teflon homogenizer in 2.5 mL of sodium chloride solution (0.9%). Afterwards, 2.5 mL of phosphate buffer (pH 7.0) and 1.5 mL of trichloroacetic acid (1.7 mol/L), were added to 0.5 mL of previously obtained homogenate. The whole sample was shaken vigorously and centrifuged for 15 min at 4000 rpm at 0°C, to separate the protein sediment. One milliliter of 2-thiobarbituric acid solution (69 mmol/L) was added to 3.0 mL of supernatant; the whole sample was shaken vigorously and heated for 15 min at boiling water bath. Three parallel samples were prepared for each tumor. The absorbance of samples was measured at $\lambda$ = 530 nm after their cooling to room temperature. The reference sample was prepared analogously with 0.5 mL of sodium chloride solution (0.9%). TBARS content was quantified in relation to fat content and to tumor tissue weight.

### Statistical analysis

All data are shown as the mean values ± standard deviation. For variables with skew distribution, data were transformed in logarithms and retransformed after calculations. Obtained results were evaluated with Statistica 9.0 (StatSoft, Poland) and GraphPad prism v.3.02 (GraphPad Software, USA). Due to the lack of normal distribution for some variables and to the relatively small number of examined tumors in some groups, the data were tested with Kruskal-Wallis test and verified with Dunn's multiple comparison test; p-value < 0.05 was considered significant.

## RESULTS

DMBA given intragastrically in a single dose of 80 mg/kg body weight was effective in the induction of mammary tumors. They appeared during the experiment in all groups and were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. The percentage of tumor-bearing animals in each group is shown in Table 1.

The higher cancer morbidity was observed in groups BOil and B2%. In those groups all animals suffered from mammary tumors. Nevertheless, we

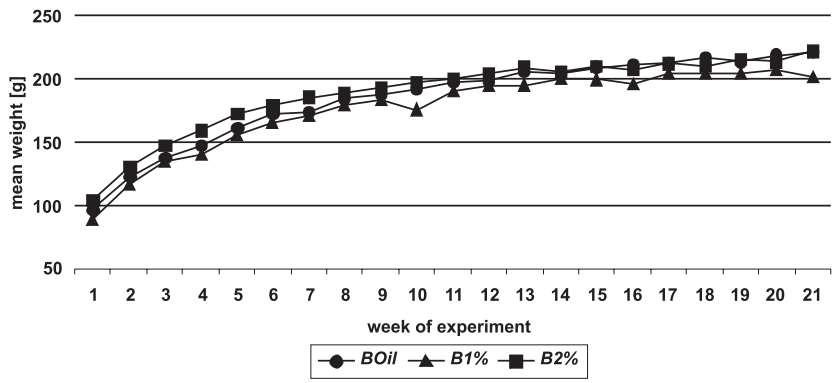


Figure 1. Changes of average body weight during the experiment of groups supplemented before DMBA administration

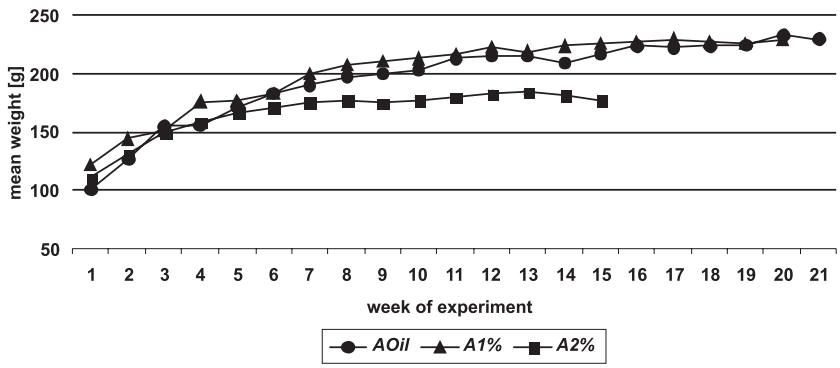


Figure 2. Changes of average body weight during the experiment of groups supplemented after DMBA administration

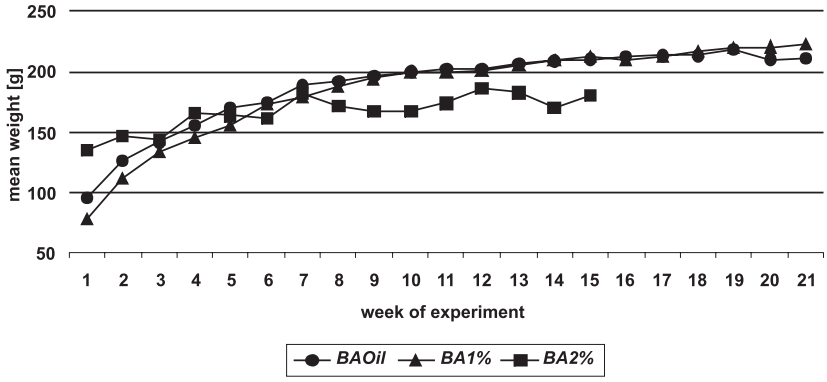


Figure 3. Changes of average body weight during the experiment of groups supplemented before and after DMBA administration

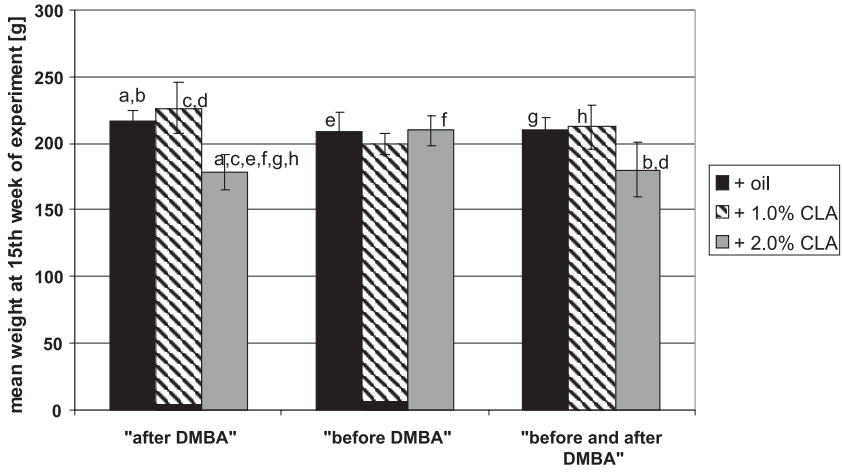


Figure 4. Mean body weight gained by each experimental group at 15th week of experiment. The same superscripts mean, that the values differ with $p < 0.05$

observed diminished induction of cancerous process in most of CLA supplemented groups. The preventive effect was the strongest in BA2% group, where breast cancer appeared only in one of nine animals. Also in A2% group the cancer morbidity was much smaller than in other groups. Furthermore, animals supplemented with the lower dose of CLA (1.0%) were less prone to chemically induced carcinogenesis than animals from similar groups receiving vegetable oil. The mammary tumors appeared and were palpated in 20-21 week of life, much later than in groups supplemented with oil. Taking into account other parameters characterizing the cancer morbidity, such as total weight of tumors and total number of tumors, we observed significant differences among all examined groups ($p = 0.0080$ and $p < 0.0010$, respectively). The preventive properties of CLA were also reflected by the diminished number and smaller size of tumors in A2% and BA2% groups.

During the whole experiment, the mean body weight of each group was checked weekly. There were no significant differences in body weight among those groups, which were fed the modified diets before the DMBA administration (Fig. 1). We observed the significant influence of diet supplementation with higher dose of CLA on body weight in A2% and BA2% groups (Figs. 2 and 3). From 7th week of the experiment, their mean body weight were much lower than body weight of other animals, which indicates that they did not utilize the forage as effectively as other groups. Moreover, the action of CLA was bound with the toxic effect of carcinogenic agent treatment and caused the exhaustion of the animals. The continuation of the experiment for A2% and BA2% group was impossible because of the cachexia of animals and for those groups the experiment was terminated at 15th week (Figs. 2 and 3).

We compared the mean weight gained at 15th week of experiment in each group (Fig. 4). There were significant differences among examined groups ($p < 0.0001$). The lowest weight gained two groups supplemented with the higher dose of CLA for a long period of time. Such body weight lowering effect was not observed with the supplementation with 1.0% dose of CLA. On the contrary, A1% group gained the highest weight of all examined groups at 15th week of experiment.

The earlier termination of the experiment for A2% and BA2% group also significantly influences the weight of internal organs, as their weights were the lowest in these two groups (Table 3). However, the applied modification of diet also had a significant impact on the organs' weight. The highest mean weight of liver was determined for BOil group, whereas A1% group gained the highest weight of kidneys and heart. As far as weight of spleen is considered, we observed great differences among individuals in all investigated groups but also significant differences among groups. The efficiency of DMBA action also modified the organs' weight, as the comparison of these parameters revealed their significantly higher values for individuals suffering from mammary tumors than in individuals without breast cancers (data not shown).

Fatty acids methyl esters (FAME) profiles in fat isolated from mammary tumors were determined using GC. In our experiment, we were able to identified 32 fatty acids in tumors. The following fatty acids were found to be the main fatty acids in the tumors of all investigated groups: palmitic C16:0, oleic C18:1 n-9 cis, linoleic C18:2 n-6 cis, arachidonic C20:4 n-6 and stearic C18:0 (Table 4).

There were significant differences in the concentration of some of the fatty acids among examined groups (Tables 4 and 5).

Two main saturated fatty acids were detected in the highest amounts in tumors from B2% group (C16:0) and BOil (C18:0), whereas their content in samples from BAOil (C16:0) and B1% (C18:0) was the lowest. In BAOil group we also detected the highest share of main monounsaturated acid - oleic acid, while the lowest concentration of this acid was characteristic for B2% group. Among polyunsaturated fatty acids, concentration of linoleic acid was the highest in fat fraction of mammary tumors but its content differed significantly among individuals in each group. We observed its highest content in tumors obtained from BAOil group. Moreover, cancerous tissues from this group were also characterized by the lowest content of arachidonic acid – the main metabolite of linoleic acid. Similar dependence was observed also for α-linoleic acid (C18:3 n-3), which content in BAOil was the highest among all investigated groups, and its metabolite - eicosapentaenoic acid (C20:5 n-3), which content in this group was the smallest. As far as docosahexaenoic acid (C22:6, n-3), the other ALA metabolite is concerned, its lowest amount was detected in adenocarcinomas from A2% and the highest – in BOil groups. The highest concentration of arachidonic acid was detected in a single analyzed tumor from BA2%.

Two main CLA isomers identified as cis-9, trans-11 CLA and trans-10, cis-12 CLA, were detected in mammary tumors from all investigated groups. Bio-C.L.A. used as the source of CLA consisted of several fatty acids, with prevailing share of two CLA isomers: trans-10, cis-12 CLA (33%) and cis-9, trans-11 CLA, rumenic acid (31%) (13). We observed that the applied dose of CLA supplement influenced their content in adenocarcinomas of mammary glands as the highest content of both CLA isomers were detected in groups supplemented with the higher dose of CLA and their content in groups supplemented with vegetable oil was minimal. Cis-9, trans-11 CLA was shown to be the predominant CLA isomer in fat from mammary tumors of all groups, while trans-10, cis-12 CLA was detected in lower amounts, although their concentration in applied diets was similar (Table 2). The highest content of rumenic acid was detected in A2% group ($1.16 \pm 0.98\%$) while trans-10, cis-12 constituted only $0.74 \pm 0.73\%$ of FA in mammary tumors from this group.

There were no significant differences in tumors fat content among examined groups, although some tendencies were observed – its content was elevated in mammary tumors obtained from groups supplemented with higher dose of CLA (Table 3). It was not possible to quantify the absolute content of CLA isomers in each group, as their amounts were below the limit of quantification. Only their percentage share in the total pool of fatty acids was evaluated. Especially in groups receiving vegetable oil or supplemented with CLA for a very short period of time, content of CLA isomers was below the quantification limit in some individuals. Moreover, in all investigated groups there were significant differences in both isomers content among individuals (Table 3). The highest concentration of rumenic acid (in fat fraction and in tissue) was determined in A2% group, whereas in BOil it was not possible to quantify its content. Adenocarcinomas of A2% were also characterized by the highest mean content of trans-10, cis-12 CLA. Furthermore, mean content of

Table 3. Comparison of mean weights of organs and fat, CLA and TBARS content in mammary tumors.

| | Groups of animals | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | AOil | A1% | A2% | BOil | B1% | B2% | BAOil | BA1% | BA2% | p value |
| Liver [g] | 6.48 ± 0.57[a] | 6.20 ± 0.91 | 5.58 ± 0.47 | 6.73 ± 1.62 | 5.63 ± 0.41 | 5.75 ± 0.61 | 6.56 ± 1.34 | 6.13 ± 0.69 | 5.35 ± 0.63[a] | 0.0013 |
| Kidneys [g] | 1.81 ± 0.07[a,b] | 1.83 ± 0.18[c,d] | 1.41 ± 0.08[a,c,e,f,g,h] | 1.71 ± 0.18[e] | 1.62 ± 0.13 | 1.74 ± 0.17[f] | 1.70 ± 0.14[g] | 1.63 ± 0.31[h] | 1.50 ± 0.16[b,d] | < 0.0001 |
| Spleen [g] | 0.81 ± 0.25[a,b] | 0.71 ± 0.23[c] | 0.42 ± 0.04[a,c,d,e,f,g,h] | 0.71 ± 0.28[d,e] | 0.61 ± 0.30 | 0.67 ± 0.14[f] | 0.76 ± 0.30[g] | 0.71 ± 0.23[h] | 0.49 ± 0.09[b] | < 0.0001 |
| Heart [g] | 0.84 ± 0.04 | 0.95 ± 0.15[a] | 0.72 ± 0.04[a,b,c,d,e] | 0.92 ± 0.09[b] | 0.88 ± 0.09[c] | 0.79 ± 0.05[f,g] | 0.93 ± 0.10[d,f,h] | 0.92 ± 0.12[e,g] | 0.79 ± 0.08[h] | < 0.0001 |
| Fat [%] | 2.27 ± 0.84 | 2.13 ± 1.25 | 2.60 ± 0.76 | 2.32 ± 1.03 | 2.30 ± 1.30 | 1.68 (0.69 – 4.14)* | 2.17 ± 1.35 | 2.52 ± 1.46 | 4.58 | 0.8079 |
| cis-9, trans-11 CLA [mg/g of fat] | 56.4 (14.0 – 227.6)* | 2287.4 ± 1030.1 | 3690.7 (1355.9 – 10045.3)* | 96.5 ± 0.00 | 78.8 ± 66.2 | 808.6 ± 749.5 | 269.6 (90.6 – 802.0)*[,a] | 3068.9 ± 2507.9[a] | 2903.8 | 0.0020 |
| cis-9, trans-11 CLA [mg/g of tissue] | 1.8 ± 1.4[a,b] | 65.9 ± 43.4 | 112.2 (22.9 – 550.7)*[,a] | 0.6 (0.0 – 13.4)* | 2.6 ± 2.3 | 22.6 ± 21.5 | 5.8 ± 2.4[c] | 63.8(16.8 – 242.4)*[,b,c] | 72.1 | 0.0003 |
| trans-10, cis-12 CLA [mg/g of fat] | | 1428.8 ± 885.3 | 4087.7 ± 3363.8 | 31.9 ± 0.0 | | 632.4 (156.7 – 2552.3)* | 265.7 (40.5 – 1742.2)* | 1654.5 ± 1457.9 | 1462.1 0 | .3018 |
| trans-10, cis-12 CLA [mg/g of tissue] | | 46.0 ± 40.1 | 85.1 (14.0 – 516.2)* | 2.7 ± 0.0 | | 15.8 ± 3.7 | 5.8 ± 3.3 | 28.1 (4.5 – 173.5)* | 35.4 | 0.1320 |
| TBARS [nmol/g of tissue] | 24.3 ± 17.6 | 20.6 ± 5.7 | 24.0 ± 15.0 | 32.1 ± 16.3 | 23.7 ± 16.4 | 17.8 ± 17.0 | 30.3 ± 17.9 | 36.2 ± 18.5 | 350.1 | 0.5042 |
| TBARS [mmol/g of fat] | 1.18 ± 1.11 | 1.17 ± 0.73 | 0.87 ± 0.30 | 1.58 ± 1.27 | 1.03 ± 0.8 | 1.20 ± 1.14 | 1.35 (0.29 – 6.19)* | 1.67 ± 0.92 | 7.64 | 0.8284 |

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration. All data are shown as the mean values ± standard deviation. For variables with skewed distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as mean and confidence interval. p value < 0.05 - significant differences among groups in Kruskal–Wallis test; values with the same superscripts differ significantly.

this isomer in fat fraction of A2% tumors was higher than the content of cis-9, trans-11 CLA, although in other investigated groups the tendency was opposite – we detected higher amounts of rumenic acid than trans-10, cis-12 CLA. The highest concentrations of CLA isomers were revealed in mammary tumors obtained from groups supplemented with 2.0% of Bio-C.L.A., which indicates the influence of applied dose on the CLA isomers content in cancerous tissues.

TBARS content in mammary tumors was determined with spectrophotometric method and was express also in terms of fat content as it is considered as an indicator of lipids peroxidation processes. Results for TBARS are summarized in Table 3. Although we detected very high concentration of TBARS in one mammary tumor of BA2% group, we found this result as an outlier and this single result was not included into statistical analysis. The comparison of TBARS content in cancerous tissues from other investigated groups did not reveal any significant differences. Moreover, we observed large diversity in results among individuals in investigated groups.

## DISCUSSION AND CONCLUSION

Some species of laboratory animals treated with carcinogenic agent become prone to cancer development. In our experiment, single DMBA treatment of Sprague-Dawley female rats at 50th day of life caused the mammary tumors appearance in all groups. They were identified as adenocarcinomas and papillary adenocarcinomas of mammary gland. Our previous experiment (14), as well as the experiments conducted by other researchers (15-18) revealed the usefulness of this model in breast cancer research. We observed the significant anticarcinogenic properties of CLA: diminished breast cancer morbidity and decreased weight and number of mammary tumors in CLA supplemented groups (Table 1). This observation is in accordance with our previous results (11, 19) and with the results of others. Corl et al. detected that CLA as well as its precursor - vaccenic acid administration decreased the mammary tumors risk in laboratory animals (20, 21). Also Ip et al. observed that 0.5%, 1.0% and 1.5% of CLA reduced the number of malign and benign breast tumors in female rats (17). Moreover, even smaller doses of CLA (0.05–0.5%) given to rats for 9 months effectively prevented the breast cancer development. They observed also that short (5 weeks) administration of CLA before DMBA treatment at 50th day of life diminished this cancer risk (17). Thompson et al., who compared 1-month supplementation with 1.0% CLA before DMBA treatment and 6-month supplementation, revealed the high effectiveness of short CLA administration followed by the carcinogenic agent administration (18). Our results – the lower cancer morbidity in B1% group, receiving 1.0% of CLA mixture only for 14 days (from 37th till 50th day of life) – confirm protective efficacy of CLA administered in early stage of life, before cancerous process induction. Ip et al. explained that CLA given early, when mammary glands maturate, cause a decrease in TEB cells' number and stimulate their differentiation (17). This brought about the decrease of the amount of potential places of cancerous process initiation, because TEB are the main places of adenocarcinomas induction in mammary glands of rodents (22). However, addition of CLA to diet after carcinogenic agent treatment also diminished the number of breast tumors and their growth (23). The activity of CLA depends on their dose in diet and its maximum is observed at 1.0%, regardless the carcinogenic agent and other dietary lipids presence (24). We observed the higher anticancer effectiveness of 2.0% CLA but this dose seems to be too high because of the cachexia stimulation. Our results ostensibly disagree with the statement of Pariza et al., who claimed that one of the CLA influence on cancerous process is through the cachexia inhibition (25). However, this effect is attributed to rumenic acid, whereas trans-10, cis-12 CLA does not demonstrate such activity. The fact, that applied preparation contained the mixture of two main CLA isomers, may cause such incompatibilities.

We observed much lower mean body weight of animals supplemented with higher dose of CLA since 7th week of experiments, what was the reason of their earlier decapitation. Many researchers emphasized the ability of CLA isomers to reduce the body mass. Especially trans-10, cis-12 CLA seems to be potent and its action has few potential mechanisms: reduction of lipoprotein lipase activity, diminished differentiation of preadipocytes into adipocytes through the genes inhibition and stimulation of lipids β-oxidation by activation of crucial enzymes expression (26, 27). Bio-C.L.A., which we used as a source of conjugated linoleic acids, consisted of few fatty acids with prevailing share of two CLA isomers: cis-9, trans-11 and trans-10, cis-12, which were present in equal amounts (Table 2). High intake of trans-10, cis-12 CLA seemed to be responsible for much lower weight gained by animals supplemented with 2.0% of CLA. Our results are in accordance with those obtained by He et al., who applied synthetic isomers of CLA to laboratory

Table 4. Fatty acids profile in mammary tumors of investigated groups.

| Fatty acid [%] | Groups of animals | | | | | | | | | Kruskal-Wallis test p value |
|---|---|---|---|---|---|---|---|---|---|---|
| | AOil | A1% | A2% | BOil | B1% | B2% | BAOil | BA1% | BA2% | |
| C12:0 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.3 | 0.5 (0.13–2.04)* | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 | 0.1908 |
| C13:0 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.1 (0.03-0.18)* | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.2 ± 0.1 | - | - | 0.23 | 0.3632 |
| C14:0 | 1.2 ± 0.3 | 1.0 ± 0.1 | 1.1 ± 0.3 | 1.1 ± 0.2 | 1.7 ± 1.0 | 3.0 ± 5.6 | 1.1 ± 0.2 | 1.1 ± 0.2 | 1.2 | 0.1058 |
| C15:0 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.3 | 0.3584 |
| C16:0 | 24.0 ± 2.0 | 23.3 ± 0.9 | 22.9 ± 1.7 | 22.1 ± 1.7 | 23.7 ± 1.0 | 24.1 ± 2.2 | 20.8 ± 1.6 | 22.9 ± 1.6 | 22.6 | 0.0093 |
| C16:1 | 2.6 ± 1.4 | 1.7 ± 0.4 | 1.2 ± 0.0 | 1.6 ± 0.6 | 1.6 ± 0.2 | 2.1 ± 0.8 | 1.4 ± 0.3 | 1.6 ± 0.7 | 1.1 | 0.1308 |
| C18:0 | 10.1 ± 2.0 | 9.2 ± 1.3 | 9.4 ± 2.2 | 10.5 ± 2.3 | 8.9 ± 1.6 | 9.4 ± 1.7 | 9.3 ± 2.1 | 10.3 ± 2.2 | 10.1 | 0.5336 |
| C18:1 n-9 *cis* | 18.7 ± 2.2 | 18.82 ± 2.46 | 20.0 ± 5.7 | 19.5 ± 2.8 | 19.6 ± 3.2 | 16.9 ± 4.3 | 23.3 ± 4.1 | 18.4 ± 2.7 | 19.4 | 0.0752 |
| C18:2 n-6 *trans* | 0.3 ± 0.1 | 0.1(0.03-0.18) | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 (0.05–0.52)* | 0.1 (0.03-0.15)* | 0.1 | 0.0018 |
| C18:2 n-6 *cis* | 12.0 ± 3.4 | 14.7 ± 5.9 | 15.0 ± 1.2 | 13.6 ± 5.5 | 16.0 ± 5.1 | 9.9 ± 5.1 | 19.1 ± 5.2 | 15.1 ± 6.5 | 18.5 | 0.0731 |
| *cis*-9, *trans*-11 CLA | 0.03 ± 0.01 | 0.5 ± 0.15 | 1.16 ± 0.98 | 0.02 ± 0.00 | 0.05 ± 0.01 | 0.04 (0.02-0.08)* | 0.05 ± 0.03 | 0.64 ± 0.34 | 0.84 | < 0.0001 |
| *trans*-10, *cis*-12 CLA | 0.02 ± 0.00 | 0.22 ± 0.12 | 0.74 ± 0.73 | 0.01 ± 0.00 | 0.01 ± 0.00 | 0.02 (0.01-0.06)* | 0.03 ± 0.02 | 0.32 ± 0.19 | 0.44 | < 0.0001 |
| C18:3 n-6 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 (0.02–0.12)* | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.0 | 0.5077 |
| C18:3 n-3 | 1.8 ± 1.1 | 2.6 ± 1.7 | 2.5 ± 1.6 | 2.0 ± 1.4 | 2.8 ± 1.2 | 1.9 ± 1.2 | 3.2 ± 1.3 | 2.2 ± 1.1 | 2.4 | 0.3291 |
| C20:1 | 0.2 ± 0.0 | 0.3 ± 0.1 | 0.7 ± 0.5 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.4 | 0.0003 |
| C20:2 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.2 ± 0.1 | 0.3 ± 0.2 | 0.3 | 0.3212 |
| C20:3 n-3 | 0.2 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.0 | 0.1 | 0.1315 |
| C20:4 n-6 | 11.8 ± 3.0 | 12.0 ± 4.2 | 11.1 ± 7.9 | 12.0 ± 4.0 | 10.4 ± 5.7 | 12.7 ± 3.6 | 8.3 ± 3.8 | 11.3 ± 4.8 | 13.9 | 0.4732 |
| C20:5 n-3 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.2 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.3 | 0.0363 |
| C21:0 | 0.4 ± 0.1 | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.1 | 0.3 ± 0.0 | 0.3 | 0.1695 |
| C22:2 | 0.1 ± 0.1 | 0.0 ± 0.0 | 0.0 (0.01-0.06)* | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.1 ± 0.1 | 0.2 ± 0.1 | 0.2 | 0.0001 |
| C22:6 n-6 | 1.6 ± 0.0 | 1.5 ± 0.4 | 1.1 ± 0.7 | 1.6 ± 0.4 | 1.4 ± 0.4 | 1.5 ± 0.4 | 1.3 ± 0.4 | 1.5 ± 0.4 | 1.4 | 0.5657 |
| C23:0 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 (0.02-0.41)* | 0.2 ± 0.2 | 0.1 (0.04–0.24)* | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.2 | 0.3951 |
| C24:0 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.0 | 0.2 ± 0.1 | 0.3 | 0.0314 |
| C24:1 | 1.1 ± 0.3 | 0.9 ± 0.0 | 0.8 ± 0.2 | 1.3 ± 0.4 | 1.3 ± 0.8 | 1.0 ± 0.3 | 0.9 ± 0.3 | 1.0 ± 0.1 | 1.0 | 0.1528 |

A Oil – group of rats supplemented with vegetable oil after DMBA administration, A 1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A 2% – group of rats supplemented with 2.0% of CLA after DMBA administration, B Oil – group of rats supplemented with vegetable oil before DMBA administration, B 1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B 2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BA Oil – group of rats supplemented with vegetable oil before and after DMBA administration, BA 1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA 2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration. All data are shown as mean values ± standard deviation. For variables with skewed distribution (*), data were transformed in logarithms and retransformed after calculations; data are shown as mean and confidence interval. $p$ value < 0.05 – significant differences among groups in Kruskal-Wallis test; percentage share of C10:0, C14:1, C15;1, C17:0, C17:1, C20:0 and C22:0 was = 0.1% and these fatty acids are not shown in the table.

Table 5. Results of Dunn's multiple comparison test for fatty acids content in mammary tumors.

| | Compared groups | Dunn test p value |
|---|---|---|
| C10:0 | A2% *vs*. BA1% | < 0.01 |
| | B2% *vs*. BA1% | < 0.001 |
| C16:0 | AOil *vs*. BAOil | < 0.05 |
| | B2% *vs*. BAOil | < 0.05 |
| C18:1 n-9 *cis* | B2% *vs*. BAOil | < 0.05 |
| C18:2 n-6 *cis* | B2% *vs*. BAOil | < 0.05 |
| C18:2 n-6 *trans* | AOil *vs*. BOil | < 0.05 |
| | AOil *vs*. B1% | < 0.05 |
| | AOil *vs*. B2% | < 0.01 |
| C20:1 | AOil *vs*. A2% | < 0.01 |
| | AOil *vs*. BA1% | < 0.01 |
| C22:2 | A2% *vs*. BA1% | < 0.05 |
| | BOil *vs*. BA1% | < 0.01 |
| | B1% *vs*. BA1% | < 0.05 |
| | B2% *vs*. BA1% | < 0.01 |
| *cis*-9, *trans*-11 CLA | AOil *vs*. BA1% | < 0.05 |
| | A1% *vs*. BOil | < 0.01 |
| | A2% *vs*. BOil | < 0.01 |
| | BOil *vs*. BA 1% | < 0.001 |
| | B2% *vs*. BA 1% | < 0.01 |
| *trans*-10, *cis*-12 CLA | AOil *vs*. BA1% | < 0.05 |
| | A2% *vs*. B1% | < 0.05 |
| | B1% *vs*. BA1% | < 0.05 |
| | B2% *vs*. BA1% | < 0.05 |
| | BAOil *vs*. BA1% | < 0.05 |

AOil – group of rats supplemented with vegetable oil after DMBA administration, A1% – group of rats supplemented with 1.0% of CLA after DMBA administration, A2% – group of rats supplemented with 2.0% of CLA after DMBA administration, BOil – group of rats supplemented with vegetable oil before DMBA administration, B1% – group of rats supplemented with 1.0% of CLA before DMBA administration, B2% – group of rats supplemented with 2.0% of CLA before DMBA administration, BAOil – group of rats supplemented with vegetable oil before and after DMBA administration, BA1% – group of rats supplemented with 1.0% of CLA before and after DMBA administration, BA2% – group of rats supplemented with 2.0% of CLA before and after DMBA administration.

animals, and observed much lower body weight in comparison with control group (28). However, Ip et al. did not detect any differences in total body weight gained by rats treated with DMBA and supplemented with different doses of CLA (0.5–1.0%) (16).

Applied modification of diet significantly influenced the weight of internal organs. These observations disagreed with those made by others researchers. After 8-week supplementation with two doses of cis-9, trans-11 CLA Turpeinen et al. did not revealed any differences in heart, liver, spleen and pancreas weight (29). Also in much longer experiment of Ip. et al., with usage of CLA mixture and carcinogenic agent, there were no differences in internal organs weight (16). However, when they gave single trans-10, cis-12 CLA to mice treated with chemical carcinogen, they observed significantly higher weight of livers, hearts and spleen. Moreover, after cis-9, trans-11 CLA application, there were no such effects (30). This may suggest that effects of two major CLA isomers are opposite. Furthermore, our results show that coexisting cancerous process significantly influences both the total body weight (especially by the mass of tumors) and internal organs weight.

As far as fatty acids profile in mammary tumors is considered, we detected significant influ-

ence of applied diet on the content of some of fatty acids. Significant differences referred only to 9 of 32 analyzed fatty acids. Many authors compared the fatty acids profile in adipose tissue of mammary glands and other tissues of animals receiving CLA. Banni et al. observed lack of influence of 1.0% CLA on linoleic acid amount in adipose tissue of mammary glands but their distinct influence on reduction of linoleic acids metabolites: C18:3, C20:3 and arachidonic acid (22). Eder et al. also detected that CLA supplementation decreased the concentration of arachidonic acid as well as all n-6 fatty acids in hepatic phospholipids (31). Experiment of Cao et al. showed that 1.0% CLA in maternal diet reduced the arachidonic acid concentration in hepatic phospholipids of progeny, whereas 2.0% CLA had the opposite effect. Moreover, the impact of above mentioned diet modification on α-linolenic concentration was opposed while the linoleic acid amount was raised in both groups (32). Influence of CLA on other n-3 and n-6 fatty acids concentration was also divers. CLA supplementation increased the content of docosahexaenoic acid and all n-3 fatty acids in hepatic phospholipids (31). However, information concerning the impact of CLA supplementation on fatty acids profile in cancerous tissues is limited. One of the causes is the shortage of sufficient amounts of experimental material. Because of very low content of many fatty acids in cancerous tissues compared with normal tissues, detection of some fatty acids is difficult. Especially in relation to n-3 fatty acids these reasons are important. Senkal et al. detected much lower content of docosahexaenoic acid and eicosapentaenoic acid in gastrointestinal tumors than in livers (33). Results obtained by Hoffman et al. also confirmed lower levels of docosahexaenoic acid in cancerous than in normal tissues (34). For CLA isomers incorporation into cancerous tissues, the tendency is opposite, as their concentration in such tissues is much higher than in normal ones (34). However, comparison of CLA content in malign and benign mammary tumors revealed the lack of significant differences among them (35) or higher CLA content in benign tumors (36). CLA are easier incorporated into triacylglycerols than in phospholipids, that is why their content in neutral lipids is higher than in phospholipids (37, 38). They are built into nucleus and cytosol and their content in cellular membranes and mitochondria is much lower. Their incorporation into cellular fractions is similar with that of monounsaturated fatty acids (34). We detected higher content of cis-9, trans-11 CLA than of trans-10, cis-12 CLA in mammary tumors. Tsuzuki et al. claim that these differences are the results of different metabolism of main CLA isomers, while only trans-10, cis-12 activates β-oxidation of fatty acids and facilitates its own metabolism (39).

Results of many studies emphasize the higher content of TBARS in serum of patients suffering from different types of cancer: breast, lungs, stomach, small intestine (7, 8). Czeczot et al. detected higher concentration of TBARS than in control tissues in all investigated types of tumors, except malign liver tumors. Obtained results indicate that malignancy and proliferation of cells are connected with low level of lipids peroxidation (40). Some authors claimed that CLA possess strong antioxidant properties. Ha et al. found CLA to be as potent antioxidant as α-tocopherol and almost as strong as butylhydroxytoluene (41). Ip et al. also confirmed that CLA can inhibit the lipids peroxidation, because the levels of malonyldialdehyde in tissues were much lower after CLA treatment (24). Furthermore, they observed the strongest anticancerogenic effect of 1.0% CLA, whereas 0.25% CLA was the most effective dose to inhibit the TBARS formation in tissues. CLA mixture given to animals caused the decrease of TBARS levels in their mammary glands (16). However, Chen et al. observed much higher concentration of TBARS in plasma of mice with gastrointestinal tumors treated with rumenic acid or trans-10, cis-12 CLA (42). Turpeinen et al. also detected much higher concentration of 8-isoPGF2α in urine of animals receiving both CLA mixture and single trans-10, cis-12 CLA, what suggests the pro-oxidative properties of CLA (29). Moreover, Stachowska et al. indicated that CLA increased formation of reactive oxygen species (ROS) in macrophages (43). According to many researchers, an increased peroxidation of lipids caused by CLA supplementation is connected with cytotoxic effect of conjugated fatty acids against cancer cells. An increased susceptibility to oxidation of n-3 polyunsaturated fatty acids is, according to many authors, the main cause of their chemopreventive properties. Products of their oxidation act toxic at cancerous cells (44). Osinsky et al., who detected higher concentration of malonyldialdehyde in tumors after administration of cobalt complexes, found this effect to be beneficial (45). We detected much higher content of TBARS in mammary tumor of BA2% group, but we did not observed any differences in concentration of TBARS among other investigated groups. Our results do not confirm neither prooxidant nor antioxidant properties of CLA. However, it seems to be advantageous to evaluate the TBARS and other biofactors content in serum or

other tissues. This will help to recognize the overall oxidation status of the organism and will explain the influence of CLA supplementation on lipids and other compounds oxidation.

Our results confirm that conjugated linoleic acids can inhibit chemically induced mammary tumors development in female rats, but their cytotoxic action seems not to be connected with lipids peroxidation. Cis-9, trans-11 CLA and trans-10, cis-12 CLA differ with their incorporation into cancerous tissues and they influence some fatty acids content in mammary tumors.

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# INTERACTIONS OF INSUMAN COMB 25 INSULIN WITH FREE RADICALS – KINETICS EXAMINATION BY ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

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**Abstract:** Insuman Comb 25 insulin's interactions with free radicals were examined in this study. The interaction of recombinant biphasic isophane insulin, Insuman Comb 25, with free radicals was assessed by continuous wave electron paramagnetic resonance (EPR) spectroscopy with microwaves of 9.3 GHz frequency. The model free radical molecule – DPPH (1,1-diphenyl-2-picrylhydrazyl) was used. EPR spectra of DPPH in contact with the tested insulin and EPR line of paramagnetic DPPH as the free radical with unpaired electrons localized on nitrogen (N) atoms, were compared. The aim of this study was to check the hypothesis about scavenging activity of Insuman Comb 25 insulin against free radicals. The EPR spectra were recorded numerically by EPR spectrometer of Radiopan (Poznań, Poland) and the Rapid Scan Unit of Jagmar (Kraków, Poland). Amplitudes (A) of EPR lines of DPPH and g-factor were analyzed. Amplitudes (A) of DPPH decreased upon contact with analyzed insulin, what confirmed its antioxidative character and scavenging activity against free radicals. The kinetics of interactions of DPPH with Insuman Comb 25 insulin was tested. Amplitudes (A) of the spectra of DPPH in contact with the insulin decreased with increasing time of interactions, and after 40 min were stabilized. It was pointed out that EPR spectroscopy may be used as the tool in pharmacy of antioxidative drugs.

**Keywords:** Insuman Comb 25 insulin, scavenging activity, free radicals, DPPH, EPR spectroscopy

Diabetes mellitus is associated with the developing risk of chronic complications (1) and deterioration of quality and shortening of life span (2, 3). Insulin therapy improve metabolic control and bring a reduction incidence of long term diabetic complications (3).

Insulin therapy is implemented in the management of patients with diabetes of all types. The need for insulin depends upon the impaired balance between insulin biosynthesis, secretion and insulin resistance (4). The use of insulin preparations is recommended for all patients with diabetes type1 (T1D) (5). The primary treatment goal of T1D is achieving and maintaining near-normoglycemia through intensive insulin therapy, avoiding acute complications, and preventing long-term complications (microangiopathy and macroangiopathy), as well as facilitating as close as possible to a normal life (6). The progressive nature of type 2 diabetes (T2D) requires clinicians to systematically evaluate patients and unfortunately first- and second-line antidiabetic agents such as metformin and the sulfonylureas do not prevent the characteristic decline in β cell function associated with T2D. Insulin replacement therapy can therefore quickly become a necessity in some patients (7). Insulin therapy is indicated for patients with T2D in whom glycemic targets were not achieved with two or more antidiabetic agents and for those who suffer from severe hyperglycemia as indicated by fasting plasma glucose (FPG) levels higher than 250 mg/dL, HbA1c concentration higher than 10% and/or symptoms of hyperglycemia (8). Available on pharmaceutical market insulin formulations are characterized by dif-

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ferent pharmacological properties with respect to time of onset, peak activity and duration of action (5).

Actually, five main types of insulin formulations are available on pharmaceutical market: regular insulin, NPH, rapid-acting analogs, basal analogs, and pre-mixed insulin that meets the different needs of patients and response to management (9, 10). Biosynthetic human insulins are still one of the most commonly used in clinical practice. Among them, biphasic premixed insulins that incorporate the combination of short or rapid-acting insulin with its intermediate-acting, cover both postprandial glucose excursion as well as basal insulin needs simultaneously. Insulin mixtures provide convenience to patients needing a simple insulin treatment plan (11, 12). As compared with basal insulin alone, premixed regimens usually tend to diminished HbA1c to a greater extent (13).

Taking into account that less costly alternative to insulin analogs - human insulins (14) - are still present on pharmaceutical market, being one of the most commonly prescribed by the clinical practitioners, we decided to evaluate the antioxidant properties of one of the human premixed formulation i.e., Insuman Comb 25, using electron paramagnetic resonance method. The interactions of Insuman Comb 25 insulin with the model free radicals (DPPH) were examined. Our study concentrated on kinetics of these interactions.

## EXPERIMENTAL

### Insulin sample

In this work, Insuman Comb 25® (suspension for injection, Sanofi-Aventis) was studied. Insuman Comb 25 insulin, produced by recombinant DNA technology, is a biphasic isophane insulin suspension consisting of 25% dissolved insulin and 75% crystalline protamine insulin.

### DPPH – the model free radical molecule

DPPH (1,1-diphenyl-2-picrylhydrazyl), which is the model free radical molecule (15, 16) was used to examine scavenging activity of Insuman Comb 25 insulin against free radicals. Chemical structure of DPPH is shown in Figure 1 (15, 16). Unpaired electron localized on nitrogen (N) atom was responsible for its paramagnetic character and for its EPR signal (15, 16).

### EPR measurements - the apparatus conditions

Electron paramagnetic resonance measurements were performed by EPR spectrometer working at the X-band of microwaves with 9.3 GHz frequency. Microwave frequency (ν) [±0.0002 GHz] was obtained by MCM101 recorder produced by EPRAD (Poznań, Poland). EPR spectrometer with continuous waves and magnetic modulation of 100 kHz produced by Radiopan (Poznań, Poland) was used. Numerical acquisition of EPR spectra of DPPH was done by the Rapid Scan Unit of Jagmar (Kraków, Poland). The time of acquisition of the single line was 1 second. The total microwave power produced by klystron was 70 mW. Attenuation of 15 dB resulted with microwave power of 2.2 mW during the measurements. This low microwave power provided guarantees of the microwave saturation absence in the signals.

### Detection of EPR spectra of DPPH

The first-derivative EPR spectra of 10% ethyl alcohol solution of DPPH and DPPH in contact with the Insuman Comb 25 insulin, were measured. The tested samples in the thin walled glass tubes with external diameter of 1 mm were located in magnetic field in the resonance cavity of the EPR spectrometer. These empty tubes did not give EPR signals in the used experimental conditions (receiver gain, microwave power, magnetic field).

The EPR spectrum of DPPH – the model free radical molecule in 10% ethyl alcohol solution was shown in Figure 2a. Interactions of Insuman Comb 25 insulin with DPPH quenched its EPR signal. This effect resulted from Insuman Comb 25 insulin scavenging activity against DPPH free radical molecules, and the antioxidant properties of this insulin.

### EPR analysis

The kinetics of interactions of Insuman Comb 25 insulin with DPPH was examined. The changes in the EPR line of DPPH during interaction with Insuman Comb 25 insulin by 5 min up to 60 min were determined. The changes of amplitudes (A) of the EPR line of DPPH with increasing of time (t) of interaction of Insuman Comb 25 insulin with DPPH

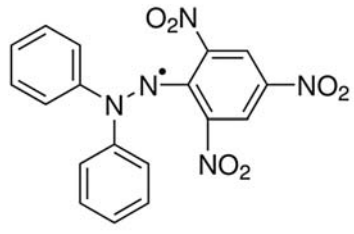


Figure 1. Chemical structure of DPPH molecule with unpaired electron (•) localized on nitrogen (N) atom (15, 16)

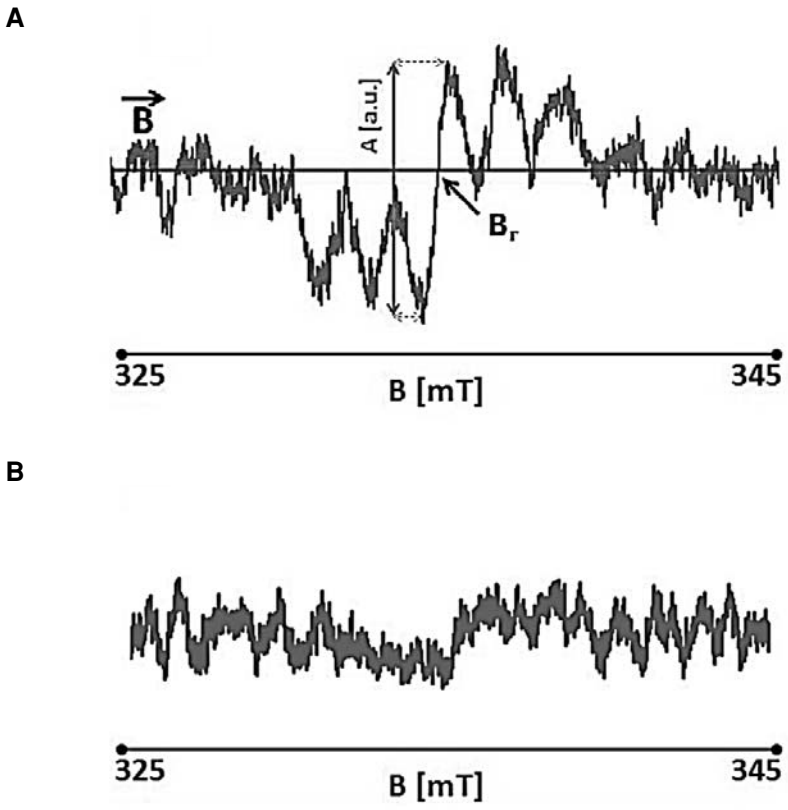


Figure 2. The first-derivative EPR spectrum of DPPH used as the model free radical molecule, where **B** = magnetic induction of the field produced by electromagnet, $\mathbf{B_r}$ = the resonance magnetic induction, **A** = amplitude (a), and DPPH interacting with the Insuman Comb 25 insulin during 60 min (b), respectively

were obtained. The decrease of amplitude (A) of EPR line of DPPH was proportional to the interactions of Insuman Comb 25 insulin with DPPH.

g-Factor depended on unpaired electron localization for EPR line of DPPH – the model free radical molecule was determined. The following formula in calculation was used (17-19): $g = h\nu/\mu_B B_r$, where: h – Planck constant, ν – microwave frequency, $\mu_B$ – Bohr magneton, $B_r$ – induction of resonance magnetic field. Microwave frequency (ν) was measured, and the resonance magnetic induction ($B_r$) was determined from the EPR line (Fig. 2a).

The following accuracies of the determined spectral parameters were obtained: [± 0.01 a.u.] for amplitudes (A), and [± 0.0002] for g-factors. The errors for the spectral parameters were determined by the method of the total differential, which respected the errors of all the measured physical values.

EPR spectra of DPPH were measured and analyzed by professional spectroscopic programs of Jagmar (Kraków, Poland) and LabVIEW 8.5 of National Instruments (USA).

## RESULTS AND DISCUSSION

Different composition and physicochemical properties of human insulin, influence the efficacy of the management process. Interactions between free radicals and insulins in terms of antioxidative properties of the former ones could modify pharmacodynamic and pharmacokinetic profile of insulin action. The results of our present study revealed that EPR spectra of DPPH free radical molecule changed after contact with Insuman Comb 25 insulin. The EPR spectra of DPPH interacting with this insulin for different times of interactions are shown in Figure 3. The influence of Insuman Comb 25 insulin on EPR spectra of DPPH confirmed our hypothesis about scavenging activity of this insulin against free radicals. The EPR spectra of DPPH and DPPH in contact with Insuman Comb 25 insulin after 60 min of their interactions, were compared in Figure 2 a,b, respectively. It is visible that the insulin quenched the EPR line of DPPH.

The results of kinetics analysis is presented in Figure 4, as the influence of time (t) of interactions with Insuman Comb 25 insulin on amplitude (A) of the EPR spectrum of DPPH free radicals. It was observed that the amplitudes (A) decreased with increasing time of interaction and after 40 min its value stabilized. The decrease of amplitude (A) reflected the scavenging properties of the model free

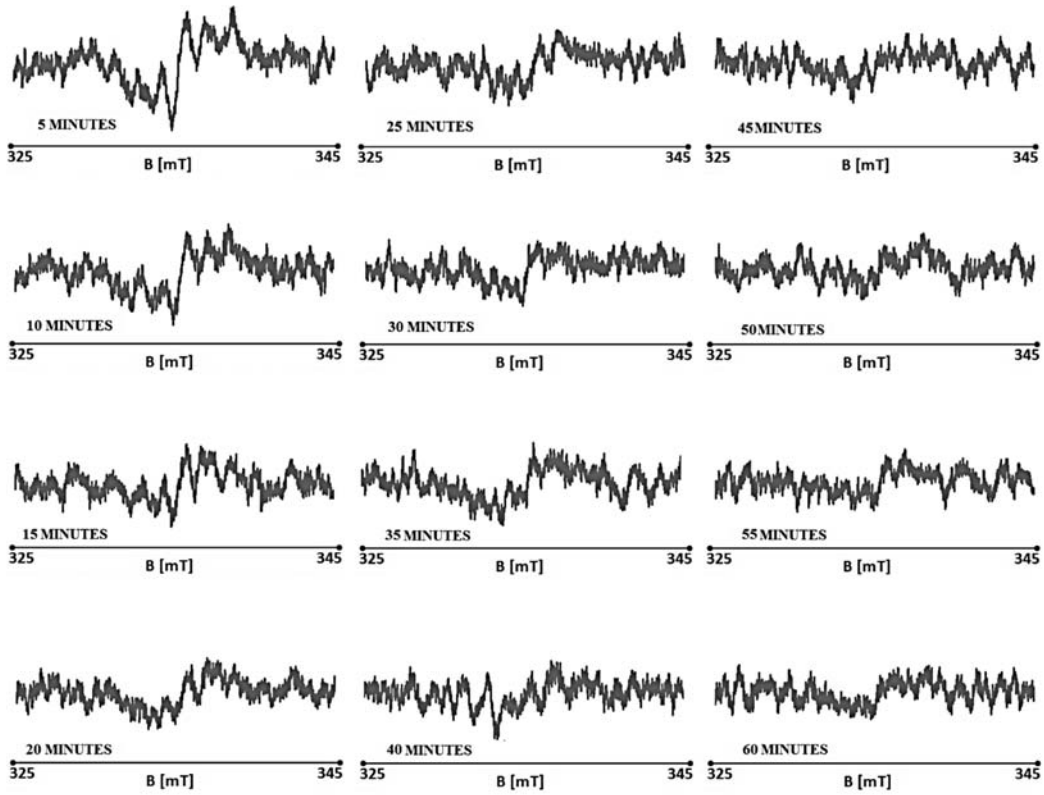


Figure 3. The EPR of DPPH interacting with the Insuman Comb 25 insulin depend on interaction time (t), respectively. **B** = magnetic induction

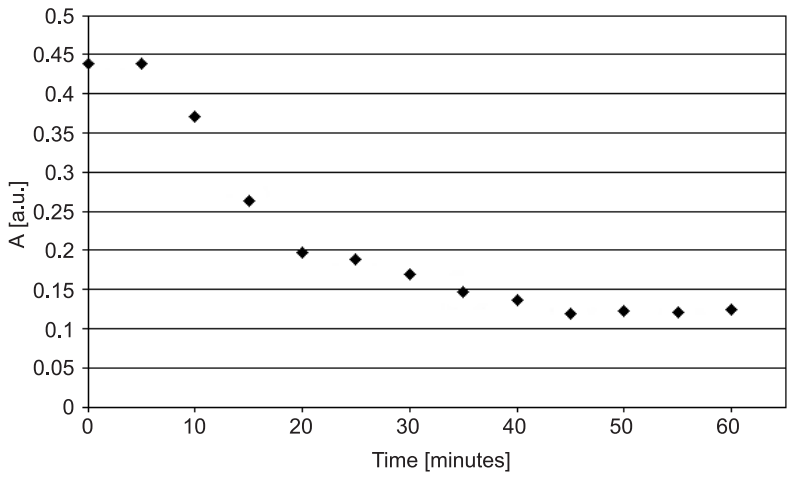


Figure 4. Changes of amplitudes (A) [± 0.01 a.u.] of the EPR spectra of DPPH in contact with Insuman Comb 25 insulin depending on the increasing time of interaction

radicals. This effect increased with increasing of the time (t), and after 40 min the scavenging was saturated. The time of saturation of scavenging activity of Insuman Comb 25 insulin pointed out that interactions of this insulin with free radicals were fast.

Our examination confirmed antioxidant character of Insuman Comb 25 insulin.

The obtained results of our examinations are difficult to discuss with similar analyses due to the lack of the results of comparable examinations of insulins' antioxidative properties assessed by EPR method. Electron paramagnetic resonance spectroscopy was proposed as the innovatory experimental technique useful in the field of pharmacy. Free radical scavenging properties of biphasic human insulin, Insuman Comb 25, can point out the benefits of introduction of insulin therapy in type 2 diabetic patients who do not achieve therapeutic goals related to effective control of blood glucose. Antioxidative properties of exogenously delivered conventional human insulins may be of great importance in reducing oxidative stress *in vivo* and delay the progression of diabetic vascular complications.

## CONCLUSIONS

Electron paramagnetic resonance (EPR) study pointed out the scavenging activity of Insuman Comb 25 insulin against free radicals. The kinetics of interactions of DPPH with Insuman Comb 25 insulin indicated that these interactions were fast and they stabilized after 40 min. The performed spectroscopic investigation by the use of microwaves proved antioxidant character of the applied insulin formulation.

**Acknowledgments**

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## DRUG SYNTHESIS

# SEMISYNTHSIS OF NOVEL SULFONAMIDES, THIOUREAS AND BIPHENYLSULFONES AS A NEW CLASS OF ANTICANCER AGENTS BY USING L-NOREPHEDRINE AS STRATEGIC STARTING MATERIAL

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**Abstract:** In continuation of our work on synthesis of novel anticancer agents, a new series of sulfonamides carrying a biologically active thiourea **3**, **4,** biphenylsulfones bearing thiourea **8**-**10** and oxazole thione **11** were designed and synthesized using L-norephedrine [phenylpropanolamine (PPA)] as strategic starting material. The synthesized compounds were evaluated *in vitro* for their anticancer activity against the human breast (MCF-7), human liver (HEPG2) and human colon (HCT116) cancer cell lines. Bisthiourea compound **8** is nearly as active as doxorubicin against (MCF-7 and HEPG2) cell lines with value ($IC_{50}$ = 6.93 and 4.0 µg/mL). Compounds **3**, **4**, **9**-**11** exhibited a moderate activity compared with doxorubicin as reference drug.

**Keywords:** l-norephedrine, sulfonamides, thiourea, biphenylsulfones, anticancer activity

Cancer is continuing to be a major health problem in developing as well as undeveloped countries (1-5). The great cancer incidence worldwide increases the search for new, safer and efficient anticancer agents, aiming the prevention or the cure of this illness. In spite of all the efforts to combat cancer, the success of the treatment of certain types of tumors has shown little progress due to their aggressiveness and the mechanisms of malignant cell metastasis. Although many classes of drugs are being used for the treatment of cancer, the need for more potent selective antitumor agents is still not precluded. From literature survey it has been reported that sulfonamides possess manifold biological activities. Representatives of this class of pharmacological agents are widely used clinically for their antibacterial (6), hypoglycemic (7), diuretic (8), antimicrobial (9), anti-carbonic anhydrase (10) and anti-thyroid activities (11). Recently, sulfonamides have been reported to show substantial anticancer activity *in vitro* and *in vivo* (12-16). E7070 (**I**) and E7010 (**II**), are examples for anticancer sulfonamides in advanced clinical trials (17) (Fig. 1). Sulfone derivatives have been found to exhibit a wide variety of pharmacological activities (18-25). Also, diphenylsulfones and bisheterocyclic compounds are reported to have a broad spectrum of biological activities. Some are endowed with antitumor (26), or antifungal properties (27). On the other hand, some pyridine and isoquinoline derivatives have various biological properties such as antimicrobial (28) and anticancer (29-32) activities. Recent studies have proved the remarkable effect of dapsone on inhibiting cell growth in glioblastoma by acting as anti-VEGF and anti-angiogenic agent *via* depriving glioblastoma of neutrophil-mediated growth promoting effects (33). Allantadapsone **III** (Fig. 1), a dapsone derivative showed high anticancer activity through inhibition of arginine methyltranseferase (PRMT1), an enzyme which plays an important role in hormone dependant cancers. A series of acylated diarylsulfone derivatives were evaluated for the same activity and compound **IV** exihibited good activity as PRMT1 inhibitor (34).

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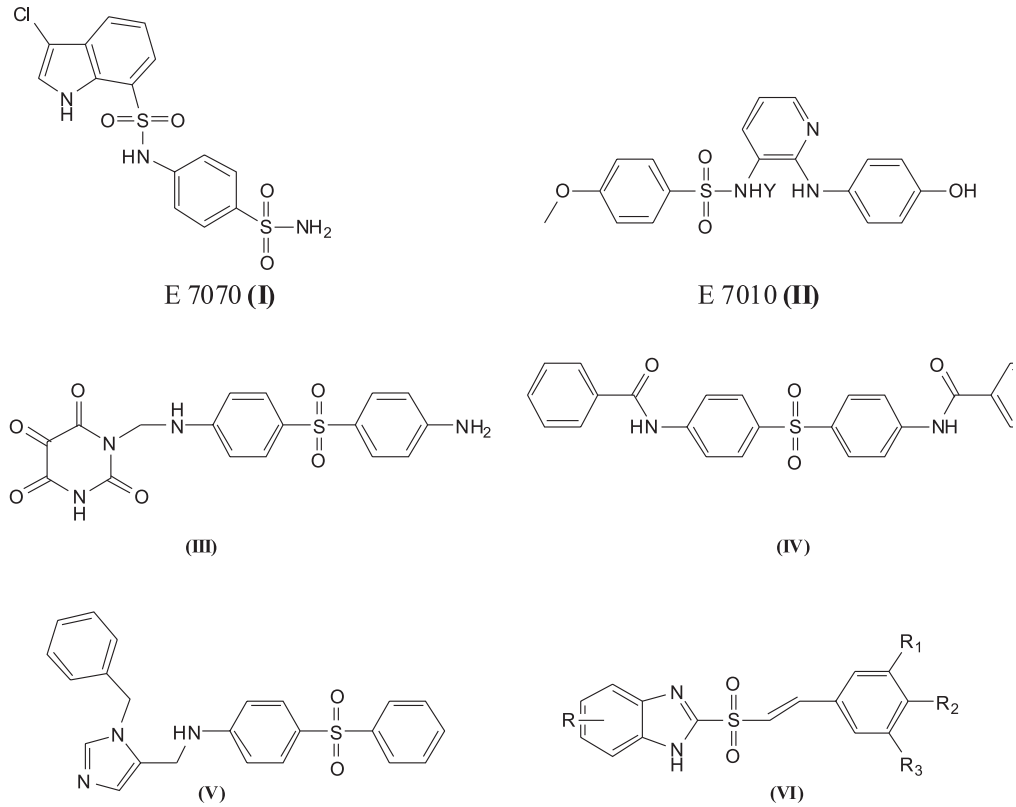



Figure 1. Sulfonamide and sulfone containing compounds in advanced clinical trials as anticancer agents

Some diarylsulfone derivatives bearing imidazole ring were evaluated for their anticancer activity through their action on inhibition of farnesyl-protein transferase (FTase), a zinc metalloenzyme which catalyzes the lipidation of 3,4-cysteine in the C-terminal tetrapeptide sequence. Compound **V** showed the lowest $IC_{50}$ as FTase inhibitor (35). Some other styryl heterocyclic sulfone derivative **VI** (Fig. 1) was interesting as anticancer agent as they block the mitogen activated protein kinase (MAPK) cascade which phosphorylates a variety of proteins including several transcription factors, which translocate into the nucleus and activate gene transcription. Negative regulation of this pathway could arrest the cascade of these events and will inhibit the proliferation of cancer cells (36, 37). Based on all these findings and as a continuation of our search in the synthesis of some novel anticancer heterocyclic compounds (38-41), the aim of this investigation was to semisynthesize some novel analogues of sulfonamide, thiourea and biphenylsulfone derivatives hoping to obtain novel compounds with significant cytotoxic activity.

## EXPERIMENTAL

### Chemistry

Reagents were obtained from commercial suppliers and were used without purification. Melting points were determined in open capillary tubes using Thermosystem FP800 Mettler FP80 central processor supplied with FP81 MBC cell apparatus, and were uncorrected. Specific rotations were measured on a Jasco P-2000 polarimeter, using a one-decimeter tube. Elemental analyses (C, H, N) were performed on a Perkin Elmer 2400 Instrument (USA). All values were within ±0.4% of the theoretical values. Infrared (IR) spectra (KBr discs) were recorded on FT-IR spectrophotometer (Perkin Elmer) at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia.$^{1}$H and $^{13}$C NMR spectra were recorded on a UltraShield Plus 500MHz (Bruker) (NMR Unite, College of Pharmacy, Salman Bin Abdulaziz University) spectrometer operating at 500 MHz for proton and 125 MHz for carbon, respectively. The chemical shift values are reported in δ (ppm) rela-

tive to the residual solvent peak, the coupling constants (*J*) are reported in Hertz (Hz). 2D-NMR experiments (COSY, NOESY, HSQC and HMBC) were obtained using standard Bruker programs. Mass spectra were run using a HP Model MS-5988 (Hewlett Packard).

**4-[3-(1-Hydroxy-1-phenylpropan-2-yl)thioureido] benzenesulfonamide (3)**

A mixture of PPA (1.51 g, 0.0l mol) and **2** (2.14 g, .0.01 mol) in chloroform (20 mL) containing a catalytic amount of triethylamine was stirred at room temperature for 5 min. The reaction mixture gave **3**; yield 98%, m.p. 188.5°C, IR (KBr, $cm^{-1}$): OH (3460), $NH_2$ and NH (3380, 3291, 3180), $SO_2$ (1381, 1156) and C=S (1271). $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.94 (d, *J* = 5.5 Hz, 3H), 4.58 (bs, 1H, C<u>H</u>-NH), 4.94 (bs, 1H, C<u>H</u>-OH), 5.63 (bs, 1H, CH-O<u>H</u>). 7.27-7.74 (m, 11H, 9 arom., $SO_2NH_2$), 8.05 (d, *J* = 7.5 Hz, 1H, CH-N<u>H</u> l-norephedrine), 9.92 (s, 1H, N<u>H</u> of sufonamide); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 12.40, 54.57, 72.96, 121.26- 127.97 (9), 138.36, 142.68, 143.16, 179.18. EIMS m/z (%): 366 (11.2, $M^+$ + 1), 365 (9.5, $M^+$), 63 (100). Analysis: calcd. for $C_{16}H_{19}N_3O_3S_2$ (365): C, 52.58; H, 5.24; N, 11.50; S, 17.55%; found: C, 52.31; H, 5.49; N, 11.29; S, 17.77%.

**1-Phenyl-2-[3-(4-sulfamoylphenyl)thioureido] propyl-2-chloroacetate (4)**

A mixture of **3** (0.365 g, 0.00l mol) and chloroacetyl chloride (0.0112 g, 0.00l mol) in chloroform (10 mL) containing 3 drops of triethylamine was stirred at room temperature for 1 h. The obtained products was purified by column chromatography (2 mm i.d., 30 g) eluted with chloroform and polarity was increased with methanol in a gradient system. Fractions of 50 mL were collected, screened by TLC and similar fractions were pooled. Fractions 9-16 afforded compound **4**. Yield: 88%, m.p. 223.8°C, IR (KBr, $cm^{-1}$): $NH_2$, NH (3405, 3330, 3205), C=O (1694), $SO_2$ (1374, 1161) and C-Cl (753). $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.98 (bs, 3H), 3.80 (s, 2H), 4.32 (d, *J* = 6 Hz, 1H, C<u>H</u>-NH), 5.04 (d, *J* = 6.8 Hz, 1H, C<u>H</u>-OH), 7.25-7.77 (m, 11H, 9 arom., $SO_2NH_2$), 8.05 (d, *J* = 7 Hz, 1H, CH-N<u>H</u> l-norephedrine), 10.49 (s, 1H, N<u>H</u> of sufonamide); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 17.55, 34.04, 54.98, 65.87, 118.63-128.50 (9), 138.36, 138.49, 141.85, 164.79, 167.38. EIMS m/z (%): 442 (29.4, $M^+$), 81 (100). Analysis: calcd. for $C_{18}H_{20}ClN_3O_4S_2$ (442): C, 48.92; H, 4.56; N, 9.51; S, 14.51%; found: C, 48.68; H, 4.29; N, 9.87; S, 14.31%.

**1,1'-(4,4'-Sulfonylbis(4,1-phenylene)-bis(3-(1-hydroxy-1-phenylpropan-2-yl)thiourea) (8)**

A mixture of biphenylsulfone isothiocyanate **7** (0.332 g, 0.001 mol) and PPA (0.302 g, 0.002 mol) in 10 mL of chloroform containing 3 drops of triethylamine was stirred at room temperature for 2 h. The reaction mixture was crystallized from MeOH to afford compound **8**. Yield: 93%, m.p. 202.3°C, IR (KBr, $cm^{-1}$): OH (3417), NH (3336, 3291), CH aromatic (3097), CH aliphatic (2954, 2863), $SO_2$ (1383, 1161), C=S (1272). $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.94 (d, *J* = 6 Hz, 3H), 4.56 (bs, 1H, C<u>H</u>-NH), 4.93 (bs, 1H, C<u>H</u>-OH), 5.62 (s, 1H, CH-O<u>H</u>), 7.26-8.11 (m, 9H, arom.), 8.12 (d, *J* = 7.5 Hz, 1H, CH-N<u>H</u> l-norephedrine), 10.02 (s, 1H, N<u>H</u> of dapsone); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 12.31, 55.03, 72.86, 121.15-127.97 (9), 135.05, 143.09, 144.34, 178.99. EIMS m/z (%): 635 (17.9, $M^+$), 182 (100). Analysis: calcd. for $C_{32}H_{34}N_4O_4S_3$ (635): C, 60.54; H, 5.40; N, 8.83; S, 15.15%; found: C, 60.18; H, 5.64; N, 8.58; S, 15.36%.

**Synthesis of compounds (8-11)**

A mixture of biphenylsulfone isothiocyanate **7** (0.332 g, 0.001 mol) and PPA (0.302 g, 0.002 mol) in 10 mL of dioxane containing 3 drops of triethylamine was heated under reflux for 2 h. The reaction mixture was cooled and purified by column chromatography (3 mm i.d., 60 g) using 10% acetone in $CHCl_3$ with few drops of acetic acid as mobile phase. The polarity was increased by increasing the proportion of acetone in a gradient system. Fractions 50 mL each were collected, screened by TLC and similar fractions were pooled. Fractions 2-6 afforded 22 mg of **11** after crystallization from MeOH, 5.73% yield. Fractions 9-11 were repurified on RP 18 column (1 mm i.d., 30 g) eluted with 25% $H_2O$ in MeOH to afford 51 mg of **10**, 12.36% yield. Fractions 13-22 (450 mg) eluted with 20% acetone in $CHCl_3$ were subjected to CPTLC (4 mm silica gel $GF_{254}$ disk, solvent: $CHCl_3$-acetone-HOAc; 85 : 15 : 0.1, v/v/v) to afford 366 mg of **8**, 67.78% yield and 63 mg of **9**, 13.59% yield.

The oxazolidine-2-thione derivative **11** was also obtained *via* reaction of PPA (0.151 g, 0.001 mol) and $CS_2$ (0.158 g, 0.002 mol) in dry pyridine (10 mL) under reflux for 30 min. The reaction mixture was dried under vacuum to give **11**.

**O-1-amino-1-phenylpropan-2-yl-4-(4-(3-(1-hydroxy-1-phenylpropan-2-yl)-thioureido)phenylsulfonyl) phenylcarbamothioate (9)**

M.p. 138.1°C, IR (KBr, $cm^{-1}$): OH (3407), NH (3386, 3310), CH aromatic (3100), CH aliphatic

(2922, 2836), $SO_2$ (1376, 1154), C=S (1238). $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.68 (d, *J* = 6.5 Hz, 3H), 0.93 (d, *J* = 6 Hz, 3H), 4.41 (bs, 1H, CH-NH), 4.55 (bs, 1H, CH-NH), 4.92 (bs, 1H, CH-OH), 5.63 (bs, 1H, CH-OH), 5.73 (d, *J* = 8.5 Hz, 1H, CH-O-), 7.26-8.11 (m, 18H, arom.), 7.92 (d, *J* = 7.5 Hz, 2H, CH-$NH_2$ l-norephedrine), 8.12 (d, *J* = 7.5 Hz, 1H, CH-NH l-norephedrine), 9.96 (s, 1H, NH of dapsone), 10.04 (s, 1H, NH of dapsone); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 12.29, 17.86, 55.02, 59.72, 72.86, 81.82, 121.14-128.40 (22), 135.05, 143.09, 144.34, 178.99. EIMS m/z (%): 635 (11.74, $M^+$), 194 (100). Analysis: calcd. for $C_{32}H_{34}N_4O_4S_3$ (635): C, 60.54; H, 5.40; N, 8.83; S, 15.15%; found: C, 60.79; H, 5.19; N, 9.12; S, 14.86%.

**1-[4-(4-Aminophenylsulfonyl)phenyl]-3-(1-hydroxy-1-phenylpropan-2-yl) thiourea (10)**

M.p. 217.7°C, IR (KBr, $cm^{-1}$): OH (3398), $NH_2$, NH (3369, 3312, 3262), CH aromatic (3055), CH aliphatic (2972, 2844), $SO_2$ (1368, 1165), C=S (1212). $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.93 (d, *J* = 6 Hz, 3H), 4.54 (bs, 1H, CH-NH), 4.93 (bs, 1H, CH-OH), 5.62 (bs, 1H, CH-OH), 6.14 (s, 2H, $NH_2$), 6.62 (d, *J* = 7 Hz, 2H), 7.26-7.42 (m, 5H, arom.), 7.54 (d, *J* = 7 Hz, 2H,), 7.71-7.75 (m, 4H, arom.), 8.12 (d, *J* = 7.5 Hz, 1H, CH-NH l-norephedrine), 9.97 (s,1H, NH of dapsone); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 12.32, 55.01, 73.83, 112.95-129.21 (14), 136.21, 136.95, 143.09, 143.54, 178.98. EIMS m/z (%): 442 (13.63, $M^+$), 149 (100). Analysis: calcd. for $C_{22}H_{23}N_3O_3S_2$ (442): C, 59.84; H, 5.25; N, 9.52; S, 14.52%; found: C, 59.54; H, 5.51; N, 9.24; S, 14.19%.

**4-Methyl-5-phenyloxazolidine-2-thione (11)**

M.p. as reported (42), $^1$H-NMR (500 MHz, DMSO-$d_6$, δ, ppm): 0.66 (d, *J* = 6.5 Hz, 3H), 4.42 (m, 1H, CH-NH), 6.00 (d, *J* = 9 Hz, 1H, CH-O), 7.26-7.46 (m, 5H, arom.), 10.19 (s, 1H, NH); $^{13}$C-NMR (125 MHz, DMSO-$d_6$, δ, ppm): 16.05, 54.87, 84.85, 126.12-128.41 (5), 134.86, 187.46.

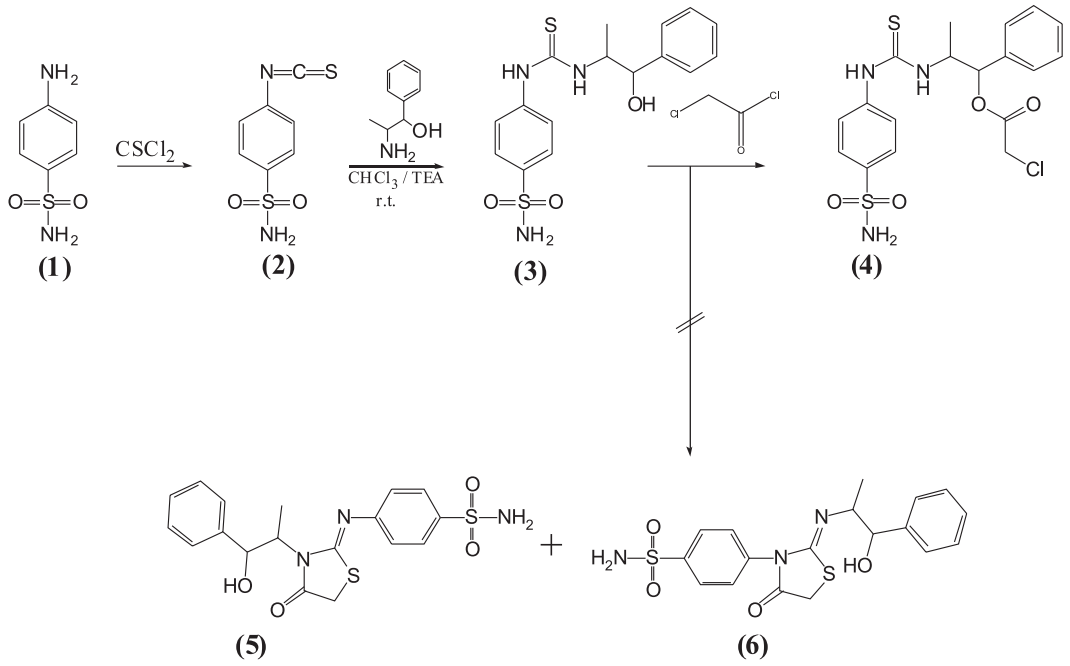
***In vitro* antitumor activity**

The cytotoxic activity was measured *in vitro* for the newly synthesized compounds using the Sulforhodamine-B stain (SRB) assay using the method of Skehan et al. (43). The *in vitro* anticancer screening was done at the Pharmacology Unit, the National Cancer Institute, Cairo University. Cells were plated in 96-multiwall microtiter plate (104 cells/well) for 24 h before treatment with the compound(s) to allow the attachment of cells to the wall of the plate. Test compounds were dissolved in DMSO and diluted with saline to the appropriate volume. Different concentrations of the compound under test (5, 12.5, 25 and 50 µg/mL) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compounds for 48 h at 37°C and in atmosphere of 5% $CO_2$. After 48 h, cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. The excess of unbound dye was removed by four washes with 1% acetic acid and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an enzyme-linked immunosorbent assay ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for breast tumor cell line after the specified time (43). The molar concentration required for 50% inhibition of cell viability ($IC_{50}$) was calculated and the results are given in Table 1. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of breast cancer cell line (MCF-7), (HepG2) and (HCT 116).

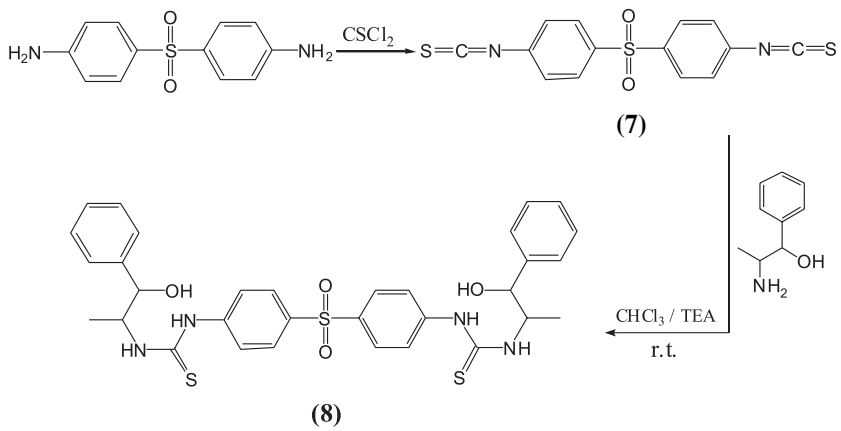
Table 1. *In vitro* anticancer screening of the newly synthesized compounds against human breast (MCF-7), liver (HEPG2), and colon (HCT 116) cancer cell lines.

| Comp. No. | $IC_{50}$ (µg/mL)[a] | | |
|---|---|---|---|
| | MCF-7 | HEPG2 | HCT116 |
| Doxorubicin | 5.40 | 2.97 | 5.26 |
| **3** | 15.2 | 22.0 | 19.9 |
| **4** | 17.1 | 15.2 | 16.7 |
| **8** | 6.93 | 4.0 | 11.5 |
| **9** | 11.2 | 14.3 | 14.4 |
| **10** | 17.0 | 14.3 | 14.4 |
| **11** | 18.7 | 11.3 | 17.8 |

[a] $IC_{50}$ value: Concentration causing 50% inhibition of cell viability




Scheme 1. Formation of compound **4**




Scheme 2. Formation of compound **8**

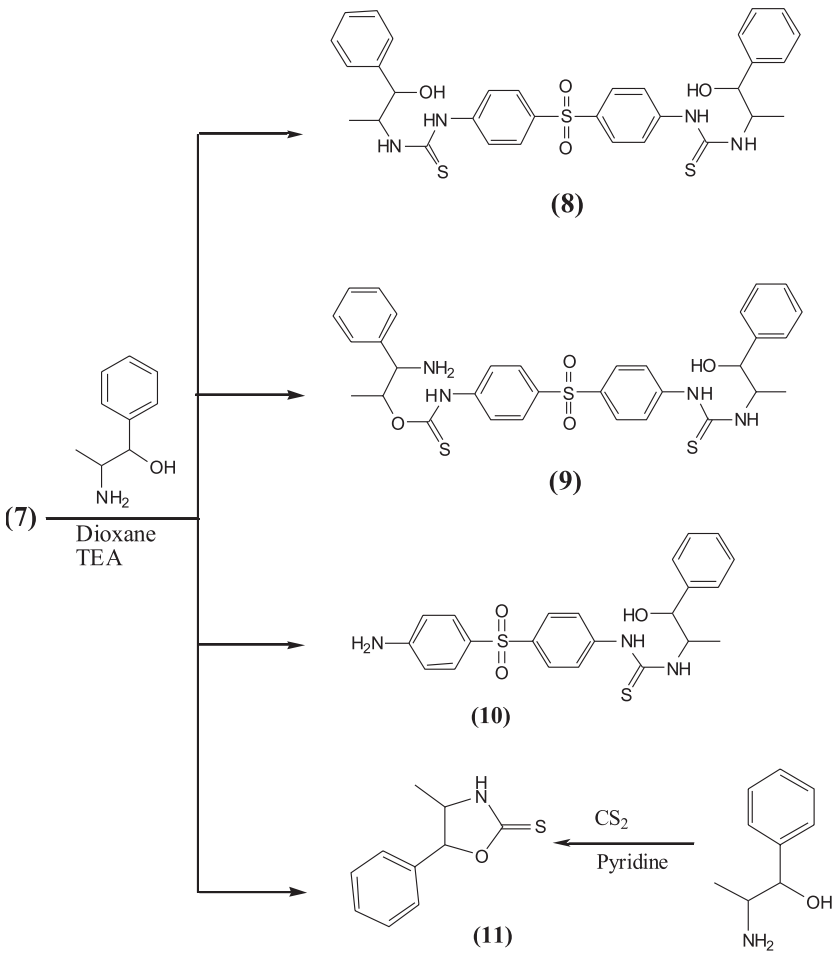
The response parameter calculated was $IC_{50}$ value, which corresponds to the concentration required for 50% inhibition of cell viability.

## RESULTS AND DISCUSSION

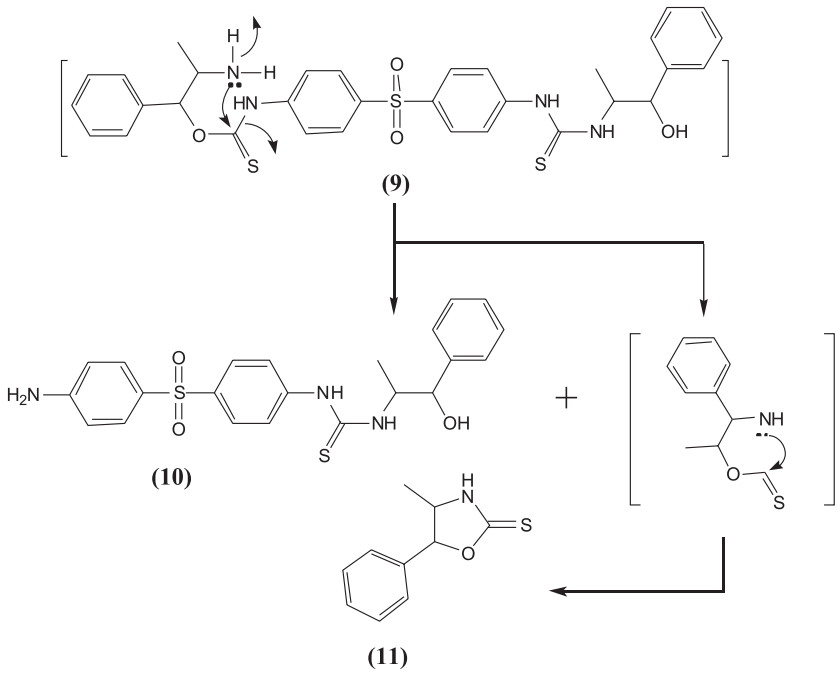
### Chemistry

This study was carried out, hoping that a group of a newly semisynthesized compounds might exhibit significant cytotoxic activity; l-norephedrine (PPA) could be a strategic intermediate for the semisynthesis of various heterocyclic systems. The number of publications on the semisynthesis and reactivity of PPA is very limited (24). This prompted the authors to undertake the semisynthesis of the corresponding thiourea **3** from the reaction of sulfonamide isothiocyanate **2** with PPA. Since PPA contains two nucleophiles: nitrogen and oxygen, it was expected to react with isothiocyanate derivatives to furnish a series of heterocyclic systems containing a

biologically active sulfonamide or biphenylsulfone moieties, which are known for their potential cytotoxic activity. The sequence of reactions leading to the formation of the target compounds is depicted in Schemes 1-4. Optical rotation of the products indicated that they retain the orientation of PPA. The behavior of PPA towards isothiocyanate was investigated .Thus, interaction of sulfanilamide isothiocyanate **2** with PPA furnish the corresponding thiourea derivative **3**, which upon reaction with chloroacetyl chloride in chloroform containing a catalytic amount of triethylamine at room temperature yielded the unexpected thioureido-propyl 2-chloroacetate derivative **4** (Scheme 1). The expected thiazolidinones **5** and **6** were eliminated from consideration on the basis of elemental analysis $^{1}$H-, $^{13}$C-NMR and mass spectral data. The IR spectrum of **3** revealed the absence of NCS band and the presence of characteristic bands for $NH_2$, NH, OH, $SO_2$ and C=S. $^{1}$H-NMR of **3** showed NH signal at $\delta_H$ 9.92 ppm assigned for S=C-NH. On the other hand, the $NH_2$ of PPA was replaced by NH at $\delta_H$ 8.54 ppm (bd, $J$ = 7 Hz) as proved by COSY and HSQC experiments. The remaining aliphatic signals were assigned to PPA moiety based on DEPT, COSY, HSQC and HMBC experiments. The region between $\delta_H$ 7.27-7.74 ppm showed signal integrated for 11 protons including 9 aromatic protons in addition to PPA protons of $SO_2NH_2$. Comparing the $^{13}$C-NMR of **3** with PPA, additional 6 aromatic resonances were observed as well as a signal at $\delta_C$ 179.18 ppm diagnostic for C=S group. The structure of **4** was confirmed on the basis of elemental analysis, IR, $^{1}$H-, $^{13}$C-NMR and mass spectral data. IR spectrum of **4** showed the presence of characteristic bands for $NH_2$, NH, C=O, $SO_2$ and C-Cl. The main difference in the NMR data of **4** compared with that of **3** is the new signals for chloroacetyl moiety at $\delta_H$




Scheme 3. Formation of compounds **9-11**




Scheme 4. Postulated formation of compounds **10** and **11**

3.80 ppm (2H) correlated with $\delta_C$ 34.04 ppm ($CH_2Cl$) in an HSQC experiment and the $\delta_C$ 167.38 ppm (C=O). The disappearance of the OH proton, the shift of the CH-O- group signal to $\delta_H$ 5.04 ppm (d, $J$ = 6.8 Hz), $\delta_C$ 65.87 ppm clearly indicated the acylation of the OH group. When biphenylsulfone isothiocyanate **7** was allowed to react with PPA in chloroform in the presence of triethylamine as catalyst at room temperature, the corresponding 1,1'-(4,4'-sulfonylbis(4,1-phenylene)-bis(3-(1-hydroxy-1-phenylpropan-2-yl)thiourea) **8** was obtained in good yield.

However, when the reaction was performed under reflux in dioxane containing triethylamine, a mixture of compounds **8**-**11** were obtained and isolated by column chromatography. The structure of compounds **8**-**11** was established on the basis of microanalytical and spectral data. IR spectrum of **8** exhibited the presence of bands for OH, NH, CH aromatic, CH aliphatic, $SO_2$ and C=S groups. $^{13}$C-NMR spectrum of **8** showed in addition to the overlapped aromatic carbons four signals at $\delta_C$ 12.31, 55.03, 72.86 and 178.99 ppm assigned for the PPA moiety and C=S group as indicated from DEPT, COSY, HSQC and HMBC experiments. The integration of the amine proton of this moiety indicated one proton because of the consumption of the second proton in the reaction with isothiocyanate group of compound **7**. Mass spectrum of compound **8** revealed a molecular ion peak m/z 635 ($M^+$, 17.9%), with a base peak at 182 (100%) consistent with the molecular formula $C_{32}H_{34}N_4O_4S_3$ indicating the dimeric nature of compound **8** where a molecule of PPA coupled to each isothiocyanate group. The $^1$H-, $^{13}$C-NMR and DEPT 1-D spectra and COSY, HSQC and HMBC 2-D spectra of **9** showed two sets of signals for PPA moieties. One set is closely related to that of **8**. However, in the other set, a clear shift in the CH-O- signals was observed ($\delta_H$ 5.73 ppm, d, $J$ = 8.5 Hz, $\delta_C$ 81.82 ppm) indicating that the reaction involved the OH group rather than $NH_2$. Mass spectrum of **9** exhibited a molecular ion peak m/z at 635 [$M^+$], (11.74%), with a base peak at 194 (100%) fully supporting the above structure. The yield of compound **9** was much less than that of the major reaction product **8**. This can be explained by the relative instability of the carbamothioate moiety in comparison with the thioureido group. Consequently, dissociation of **9** takes place leading to the formation of **10** and **11**. The mechanism of this dissociation involved the attack of the lone pair of electron of the PPA nitrogen atom on the highly positive carbon atom of the thione moiety leading to the cleavage of the thioamide bond and formation of compounds **10**. The released PPA thioate intermediate undergoes cyclization to form the oxazolidine-2-

thione derivative **11** (42) (Scheme 4). IR spectrum of **10** showed characteristic bands for OH, NH, $NH_2$, CH aromatic, CH aliphatic, $SO_2$ and C=S groups. In both $^1$H-, $^{13}$C-NMR DEPT, COSY and HSQC spectra of **10**, the signals of the PPA moiety are closely related to that of **8**. A key signal of an $NH_2$ group at $\delta_H$ 6.14 ppm indicated that one side of biphenylsulfone is bearing a primary amine after the release of the PPA moiety. The chemical shift of signals of the oxazolidine-2-thione ring of **11** were: $\delta_H$ 4.42 ppm (m), $\delta_C$ 54.87 ppm (CH-N), $\delta_H$ 6.00 ppm (d, $J$ = 9 Hz), $\delta_C$ 84.85 ppm (CH-O), $\delta_H$ 10.19 ppm (s, NH), $\delta_C$ 187.46 ppm (C=S). Assignments were performed based on COSY and HSQC experiments.

***In vitro* cytotoxic activity**

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast (MCF-7), liver (HepG2) and colon (HCT 116) cancer cell lines. The clinically used drug doxorubicin, one of the most effective anticancer agents, was used as the reference drug in the current study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of the three tested human cell lines. The data were expressed as the $IC_{50}$ values, which corresponds to the concentration required for 50% inhibition of cell lines viability. The obtained $IC_{50}$ values are presented in Table 1 indicating the *in vitro* anticancer activity of the tested compounds compared to the reference drug. It was found that, in the negative control, solvent has no effect on the cells as the surviving fraction is 1.00. The bisbiphenylsulfone **8** carrying the biologically active thiourea moiety with $IC_{50}$ values (6.93, 4.0 µg/mL) is nearly as active as doxorubicin with $IC_{50}$ values (5.40, 2.97 µg/mL) as reference drug against both breast and liver cancer cell lines. On the other hand, compound **8** exhibited a moderate activity with $IC_{50}$ value (11.50 µg/mL) against colon cancer cell line compared to doxorubicin. Compounds **3**, **4**, **9**, **10** and **11** showed weaker activity than the positive control. In an attempt to correlate the activity of **8** with its unique structural features among the other tested compounds it was noticed that: two free hydroxyl groups, two thiourea moieties and the molecular symmetry are major features in **8** rather than all the other members of the tested compounds. More *in vitro* and *in vivo* biological evaluation are required in order to explore the possibility of using this promising compound in practice.

## CONCLUSION

The objective of the present study was to semi-synthesize and investigate the anticancer activity of some novel sulfonamide and bis-biphenylsulfone carrying the biologically active thiourea moieties. Compounds **8** is nearly as active as doxorubicin as reference drug against breast and liver cancer cell line, while it was exhibited a moderate activity against colon cancer cell line. In addition, compounds **3**, **4**, **9**, **10** and **11** revealed weak activity.

**Acknowledgment**

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# SYNTHESIS AND ANTITUMOR SCREENING OF SOME NEW 2,6-BIS PYRIDINES FUNCTIONALIZED WITH PYRAZOLE-BASED HETEROCYCLES

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**Abstract:** Several new pyrazole, 1,3,4-oxadiazole, 1,2,4-triazole, 1,3,4-thiadiazole and thiazol-2-ylidene derivatives attached to pyridine ring at 2,6-positions have been synthesized starting from the versatile 3,3'-(pyridine-2,6-diyl)bis(1*H*-pyrazole-4-carbohydrazide). The newly synthesized compounds were evaluated for their *in vitro* anticancer activity against HEPG2, A549 and MCF-7 human cancer cell lines. The results showed that the newly synthesized compounds displayed low to moderate activity against the tested human cancer cell lines.

**Keywords:** pyridine, thiazole, bis-pyrazole, 1,2,4-triazole, 1,3,4-thiadiazole, anticancer screening

Several nitrogen-containing heterocyclic compounds incorporating pyridine nucleus were found to possess interesting pharmacological activities. For example, several pyridine derivatives were found to have potential anticancer activity (1-4). Moreover, 2-pyridone derivatives have considerable pharmacological importance as cardiotonic agents, such as milrinone, and as potential HIV-1 specific transcriptase inhibitors (5, 6). On the other hand, diverse pharmacological activities have been associated with pyrazole derivatives that include: antitumor, anti-inflammatory, analgesic, anti-rheumatic and antipyretic properties (7-10).

In view of these observations and in continuation of our current interest in the synthesis of a variety of substituted heterocycles for biological screening and our interest in the chemistry of 2,6-di-substituted pyridine derivatives (11-20), the present work was undertaken to synthesize some new 2,6-disubstituted pyridines bearing different heterocycles and to evaluate their antitumor activity.

## RESULTS AND DISCUSSION

### Chemistry

Recently, we have reported the preparation of the starting material for this study: 3,3'-(pyridine-2,6-diyl)bis(1*H*-pyrazole-4-carbohydrazide) (**1**) by hydrazinolysis of 2,6-bis(4-ethoxycarbonyl-1*H*-pyrazol-5-yl)pyridine (12). Treatment of **1** with carbon disulfide and potassium hydroxide, followed by acidification using HCl solution, afforded 2,6-bis[4-(5-mercapto-1,3,4-oxadiazol-2-yl)-1*H*-pyrazol-3-yl]pyridine (**2**) (Scheme 1). The bis-carbohydrazide **1** was treated also with phenyl isothiocyanate and with methyl isothiocyanate, in refluxing ethanol and afforded the corresponding thiosemicarbazide derivatives **3a** and **3b**, respectively (Scheme 1).

Heating of the thiosemicarbazide derivative **3a** in 2% sodium hydroxide solution, afforded 2,6-bis[4-(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1*H*-pyrazol-3-yl]pyridine (**4**). When **3a** was treated with sulfuric acid, it afforded the corresponding 2,6-bis[4-(5-phenylamino-1,3,4-thiadiazol-2-yl)-1*H*-pyrazol-3-yl]pyridine (**5**) (Scheme 1). The structure of compounds **3a,b**, **4** and **5** was established on the basis of their elemental analysis and spectral data.

The thiosemicarbazide derivative **3a** was treated with phenacyl chloride or chloroacetone, in the presence of catalytic amount of triethylamine, and afforded the corresponding 3*H*-thiazol-2-ylidene-3-carboxylic acid hydrazide derivatives **7a** or **7b**, respectively (Scheme 2).

Similarly, **3a** reacted also with ethyl chloroacetate, in the presence of catalytic amount of triethylamine to afford pyridine-2,6-bis[1*H*-pyrazol-3-yl-4-

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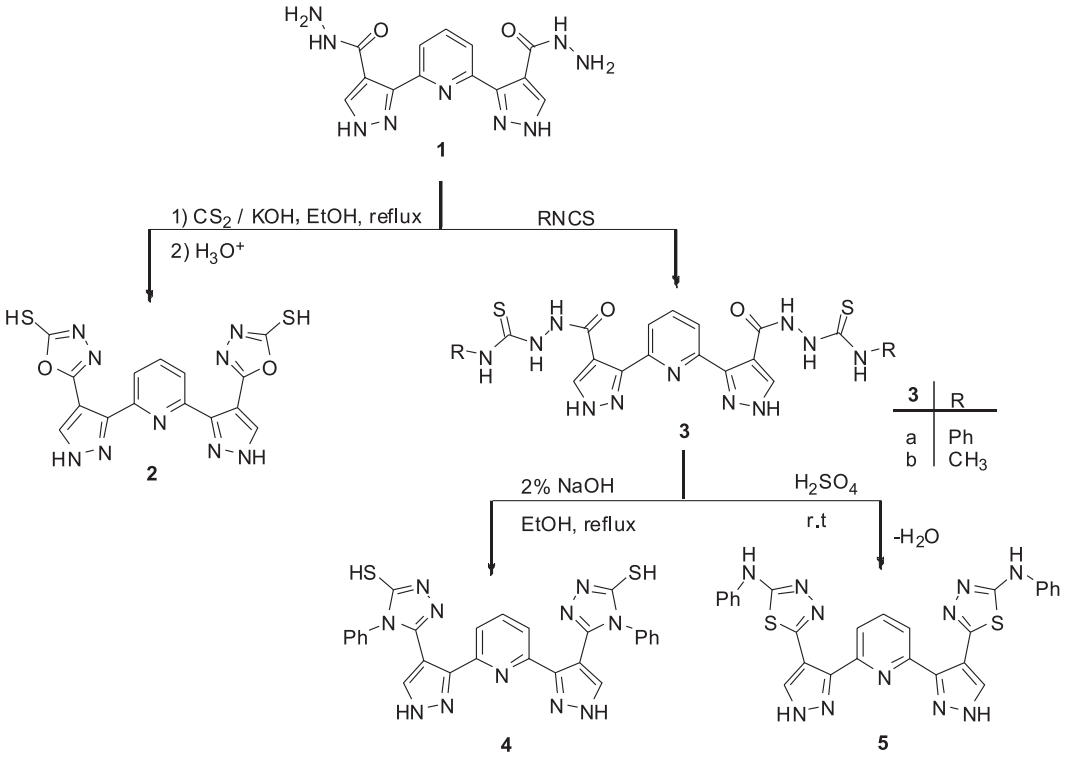



Scheme 1. Synthetic pathways for the formation of compounds **2**-**5**

(3-phenyl-4-oxo-3*H*-thiazol-2-ylidene)-3-carboxylic acid hydrazide] (**9**) *via* the non-isolable intermediate **8** (Scheme 2). The structures of the isolated products were established and confirmed on the bases of their elemental analyses and spectroscopic data (see Experimental).

Treatment of the acid hydrazide (**1**) with pentane-2,4-dione and with ethyl 3-oxobutanoate, in refluxing ethanol, afforded products identified as 2,6-bis[4-(3,5-dimethyl-1-carbonyl)-1*H*-pyrazol-3-yl]pyridine (**10**) and 2,6-bis[4-(3-methylpyrazol-5-one-1-carbonyl)-1*H*-pyrazol-3-yl]pyridine (**11**), respectively (Scheme 3).

Treatment of the acid hydrazide **1** with acetone and acetophenone, in refluxing ethanol, in the presence of few drops of glacial acetic acid, afforded the corresponding hydrazone derivatives **12a** and **12b**, respectively, on the basis of elemental analysis and spectral data (see Experimental). Finally, the acid hydrazide **1** undergoes condensation also with aromatic aldehydes *viz*. benzaldehyde, 4-methylbenzaldehyde and 4-methoxybenzaldehyde, in refluxing EtOH, in the presence of glacial acetic acid, to afford the corresponding hydrazone derivatives **13a-c** (Scheme 3).

The structure of the product **12a**,**b** and **13a-c** were established on the basis of their elemental analysis and spectral data (see Experimental).

***In vitro* anticancer screening**

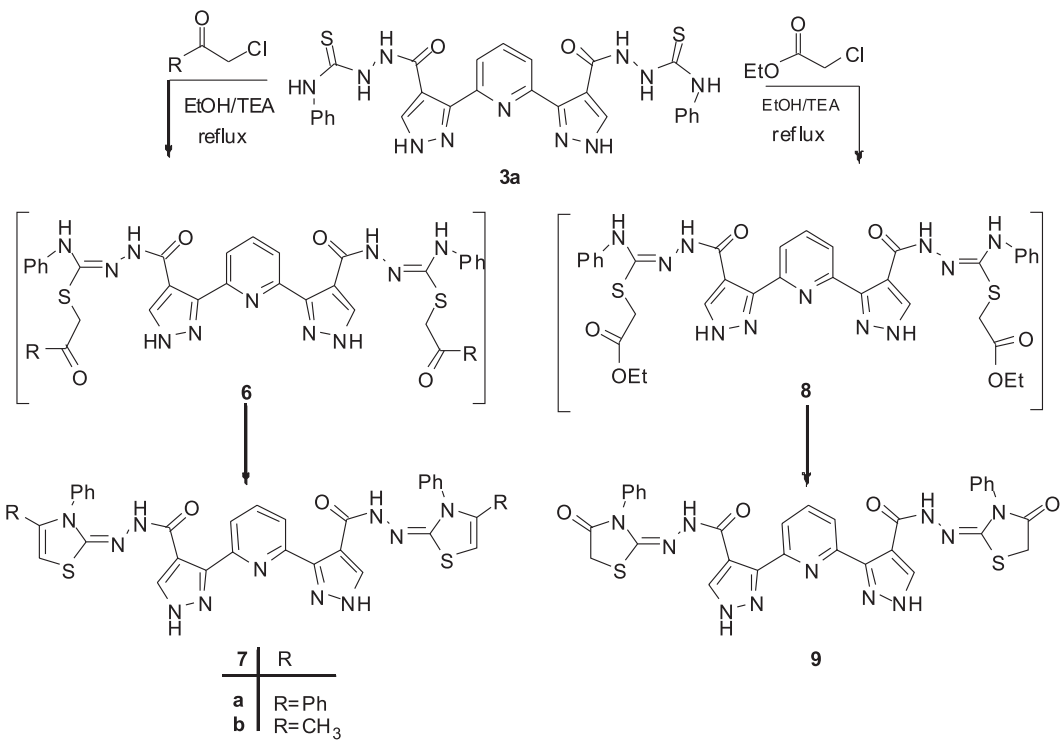
The newly synthesized compounds were preliminarily screened for their *in vitro* cytotoxic activity against 3 human tumor cell lines such as human lung adenocarcinoma (A549), hepatocellular carcinoma (HEPG2) and breast adenocarcinoma (MCF7). Table 1 shows the results of *in vitro* cytotoxic activity of the newly synthesized compounds at concentration of 100 µM where two compounds revealed anticancer activity percentage > 75% against human lung adenocarcinoma (A549).

Tow compounds that gave cytotoxic activity > 75% inhibition of cell viability at concentration (100 µM) were submitted to calculate their $IC_{50}$ value, which corresponds to the concentration required for 50% inhibition of cell viability, using MTT method (21, 22) (Table 2). Doxorubicin (one of the most effective anticancer agents) was used as reference drug. From the results obtained (Table 2), it was observed that the tested compounds showed low to moderate activity against the tested human cancer cell lines.

Table 1. Anticancer screening of the newly synthesized compounds against human tumor cell lines (A549, MCF7 and HEPG2) at concentration of 100 µM.

| Compound | *In-vitro* cytotoxic activity percentage at 100 µM (mean ± SE) | | |
|---|---|---|---|
| | A549 | MCF7 | HEPG2 |
| **1** | 35.3 ± 0.12 | *NA* | 28.8 ± 0.1 |
| **2** | 30.1 ± 0.41 | *NA* | *NA* |
| **3** | 76.5 ± 0.57 | *NA* | 44.5 ± 0.5 |
| **4a** | *NA* | *NA* | 19.3 ± 0.8 |
| **4b** | 64.5 ± 0.2 | *NA* | 15.5 ± 0.2 |
| **5** | NA | *NA* | *NA* |
| **6** | 29.9 ± 0.7 | *NA* | *NA* |
| **8a** | *NA* | *NA* | *NA* |
| **8b** | *NA* | *NT* | 19.3 ± 0.8 |
| **11** | *NA* | 13.2 ± 0.2 | 39.6 ± 0.2 |
| **12** | 60.9 ± 0.5 | *NA* | 27.7 ± 0.5 |
| **13a** | *NA* | *NA* | *NT* |
| **13b** | 49.7 ± 0.3 | *NA* | 25.3 ± 0.2 |
| **15a** | 86.1 ± 0.3 | 12.9 ± 0.3 | 52.1 ± 0.3 |
| **15b** | 19.2 ± 0.4 | *NA* | 21.4 ± 0.4 |
| **15c** | 25.6 ± 0.2 | *NA* | 13.8 ± 0.2 |
| Doxorubicin | 99.9 ± 0.2 | 91.5 ± 0.1 | 95.7 ± 0.2 |

*NA* = compounds having cytotoxic activity percentage less than 10%; *NT* = not tested.




Scheme 2. Synthetic pathway for the formation of compounds **7a**,**b** and **9**

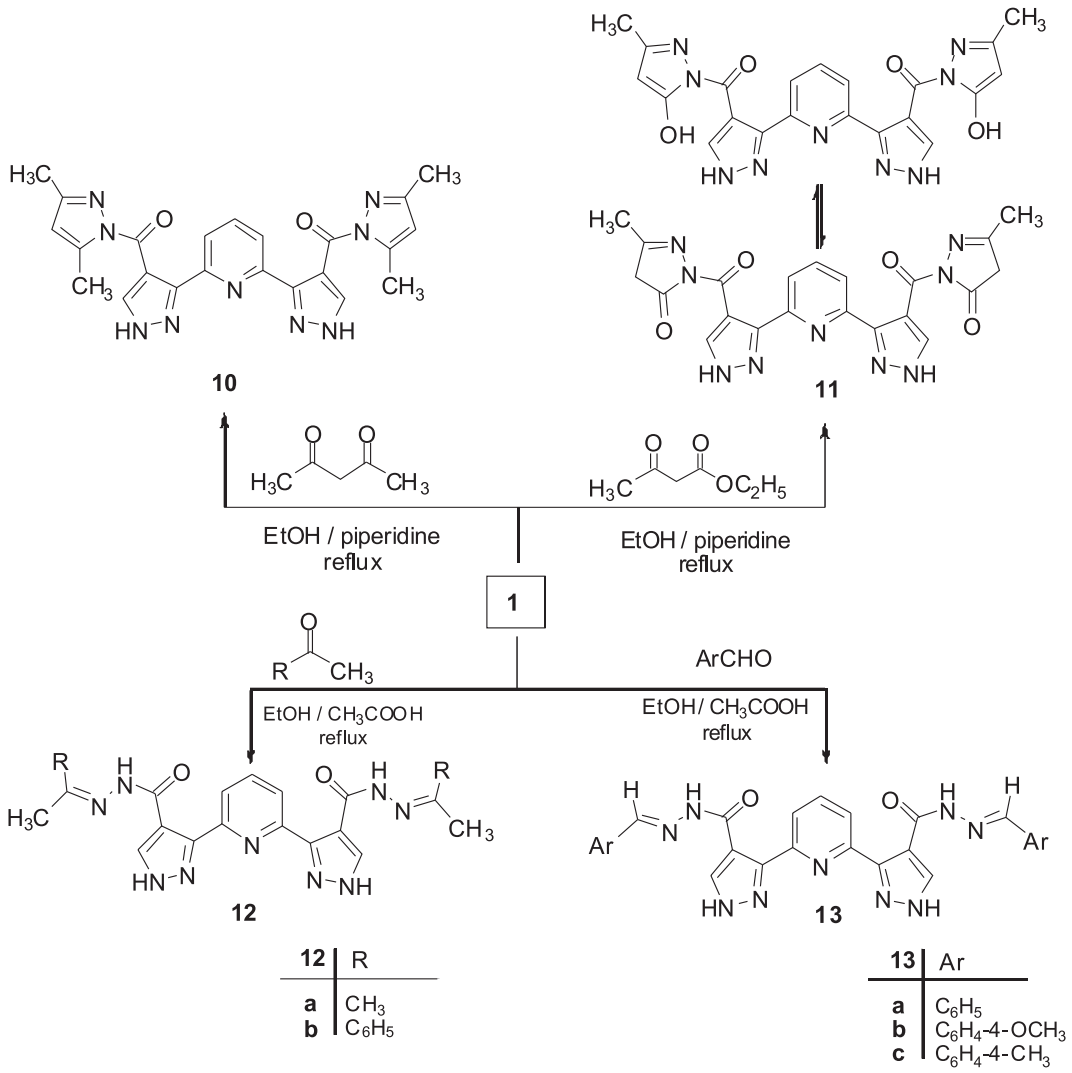
## EXPERIMENTAL

### Chemistry

All melting points were measured on a Gallenkamp melting point apparatus. The infrared spectra were recorded in potassium bromide discs on a Pye Unicam SP 3-300 and a Shimadzu FT IR 8101 PC infrared spectrophotometers. The NMR spectra were recorded on a Varian Mercury VXR-300 NMR spectrometer. $^{1}$H NMR (300 MHz) and $^{13}$C NMR (75.46 MHz) determinations were run in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-$d_6$). Chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCMS-QP1000 EX mass spectrometer at 70 eV. Elemental analyses were carried out at the Micro-analytical Centre of Cairo University, Giza, Egypt and recorded on Elementar-Vario EL (Germany) automatic analyzer. All reactions were followed by TLC (silica gel, aluminum sheets 60 $F_{254}$, Merck). 3,3'-(Pyridine-2,6-diyl)bis(1*H*-pyrazole-4-carbohydrazide) (**1**) (12) was prepared as reported in the given reference.

### 2,6-Bis[4-(5-mercapto-1,3,4-oxadiazol-2-yl)-1*H*-pyrazol-3-yl]pyridine (2)

To a stirred solution of the acid hydrazide **1** (0.71 g, 2 mmol) and potassium hydroxide (0.34 g, 6 mmol) in EtOH (20 mL), carbon disulfide (0.46 g, 6 mmol) was added gradually. The reaction mixture was stirred at room temperature for 1/2 h then refluxed for 2 h. A yellow precipitate of the corresponding potassium salt was separated. Dry ether (30 mL) was added to complete the precipitation of the formed salt which was fil-




Scheme 3. Synthetic pathways for the formation of compounds **10**, **11**, **12a**,**b**, **13a-c**

Table 2. Anticancer activity ($IC_{50}$[a], μM) of compounds **2** and **13a** against human cancer cell line (A549).

| Compound No. | Cytotoxicity [b] | | | | | | $IC_{50}$ |
|---|---|---|---|---|---|---|---|
| | 100 (μM) | 50 (μM) | 25 (μM) | 12.5 (μM) | 6.25 (μM) | 3.13 (μM) | |
| **3** | 76.3 | 29.4 | 10.7 | 2.1 | 0 | 0 | 23.2 |
| **15a** | 86.1 | 38.7 | 26.7 | 15.7 | 8.6 | 3.4 | 27.8 |
| Doxorubicin | 100 | 100 | 100 | 100 | 100 | 65.7 | 12.6 |

[a] $IC_{50}$ = compound concentration required to inhibit tumor cell proliferation by 50%. [b] Values are means of three experiments.

tered off and washed with dry ether (50 mL). The precipitated product was dissolved in cold water then acidified with HCl solution (2 M) to pH 5. The precipitated solid was filtered off, washed with water, dried, and finally recrystallized from EtOH to afford yellow crystals of 2,6-bis(4-(5-mercapto-1,3,4-oxadiazol-2-yl)-1*H*-pyrazol-3-yl)pyridine (**2**). Yield (0.63 g, 77%); yellow crystals; m.p.: 238-240°C. IR (KBr, $cm^{-1}$): 3425-3300 (NH). $^1$H NMR (DMSO-$d_6$, δ, ppm): 7.95-8.07 (m, 3H, pyridine), 8.12 (s, 2H, pyrazole H-5), 10.04 (br s, 4H, $D_2O$-exchangeable, 4NH), 13.51 (s, 2H, $D_2O$-exchangable, 2SH), MS *m*/*z* (%): 413 (12), 411 [$M^+$] (5), 229 (5), 188 (12), 171 (23), 135 (5), 129 (51), 75 (25), 60 (100). Analysis: calcd. for $C_{15}H_9N_9O_2S_2$ (411.42): C, 43.79; H, 2.20; N, 30.64%; found: C, 43.72; H, 2.29; N, 30.63%.

**Synthesis of the thiosemicarbazide derivatives (3a,b)**

***General procedure***

A mixture of (**1**) (0.65 g, 2 mmol) and phenyl isothiocyanate or methyl isothiocyanate (4 mmol) in EtOH (20 mL) was refluxed for 8-10 h and then left to cool to room temperature. Fine crystals of the thiosemicarbazide derivatives **3a** and **3b** were separated out, filtered off, washed with EtOH, dried and finally recrystallized from EtOH. The synthesized compounds together with their physical and spectral data are listed below:

**2,6-Bis[4-(3-phenylthioureidocarbamoyl)-1*H*-pyrazol-3-yl]pyridine (3a)**

Yield (0.92 g, 77%); yellow crystals; m.p.: 178-179°C. IR (KBr, $cm^{-1}$): 3227-3112 (NH, overlapped), 1670 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 5.37 (br s, 2H, $D_2O$-exchangeable, 2NH), 7.65-8.05 (m, 3H, pyridine and 10 Ar-H), 8.11 (s, 2H, pyrazole-CH-5), 10.51, 12.5, 13.7 (br s, 6H, $D_2O$-exchangeable 6NH). MS *m*/*z* (%): 597 [$M^+$] (36), 149 (14), 106 (44), 83 (57), 69 (100). Analysis: calcd. for $C_{27}H_{23}N_{11}O_2S_2$ (597.67): C, 54.26; H, 3.88; N, 25.78%; found: C, 54.33; H, 3.81; N, 25.72%.

**2,6-Bis[4-(3-methylthioureidocarbamoyl)-1*H*-pyrazol-3-yl]pyridine (3b)**

Yield (0.70 g, 74%); colorless crystals; m.p.: 214-216°C. IR (KBr, $cm^{-1}$): 3228-3209 (4NH, overlapped), 1654 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 2.41 (s, 6H, $2CH_3$), 6.31 (br s, 2H, $D_2O$-exchangeable, 2NH), 7.85-8.11 (m, 3H, pyridine), 8.14 (s, 2H, pyrazole-H-5), 10.48, 13.2, 14.2 (br s, 6H, 6NH, $D_2O$-exchangeable). MS *m*/*z* (%): 473 [$M^+$] (5), 196 (14), 166 (16), 149 (100), 121 (35), 105 (30), 77 (15), 65 (40). Analysis: calcd. for $C_{17}H_{19}N_{11}O_2S_2$ (473.54): C, 43.12; H, 4.04; N, 32.54%; found: C, 43.21; H, 4.14; N, 32.52%.

**2,6-Bis[4-(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1*H*-pyrazol-3-yl]pyridine (4)**

The thiosemicarbazide derivative **3a** (0.6 g, 1 mmol) was refluxed in sodium hydroxide solution (2%, 4 mL) for 4 h and the resulting solution was treated with charcoal then filtered. The cold filtrate was acidified with hydrochloric acid (2 M) to pH 5 and the formed solid was filtered off, washed several times with distilled water, dried and finally recrystallized from EtOH/dioxane to afford 2,6-bis(4-(5-mercapto-4-phenyl-4*H*-1,2,4-triazol-3-yl)-1*H*-pyrazol-3-yl)pyridine (**4**) in 71% yield (0.39 g); m.p. > 300°C. IR (KBr, $cm^{-1}$): 3461-3200 (2NH,). $^1$H NMR (DMSO-$d_6$, δ, ppm): 6.69-8.07 (m, 13H, pyridine and Ar-H), 8.12 (s, 2H, pyrazole-H-5), 10.51 (br, s, 2H, $D_2O$-exchangeable, 2NH), 14.21 (s, 2H, $D_2O$-exchangeable, 2SH). MS *m*/*z* (%): 561 [$M^+$] (2), 495 (15), 176 (11), 104 (22), 77 (100). Analysis: calcd. for $C_{27}H_{19}N_{11}S_2$ (561.64): C, 57.74; H, 3.41; N, 27.43%; found: C, 57.83; H, 3.32; N, 27.42%.

**2,6-Bis[4-(5-phenylamino-1,3,4-thiadiazol-2-yl)-1*H*-pyrazol-3-yl]pyridine (5)**

To an ice-cold stirred solution of the thiosemicarbazide **3a** (0.60 g, 1 mmol) in EtOH (5 mL), sulfuric acid (3 mL) was carefully added over a period of 15 min. Stirring was maintained at room temper-

ature for 4 h, then the reaction mixture was poured onto an equal volume of ice water. The formed precipitate was filtered off, washed with water several times, dried and finally recrystallized from EtOH. Yield (0.39 g, 70%); green powder; m.p.: 158-159°C. IR (KBr, $cm^{-1}$): 3410, 3215 (NH). $^1H$ NMR (DMSO-$d_6$, δ, ppm): 6.99-8.11 (m, 13H, pyridine and Ar-H), 8.35 (s, 2H, pyrazole-H-5), 10.40 and 11.43 (br s, 4H, $D_2O$-exchangeable, 4NH). MS *m/z* (%): 561 [$M^+$] (13), 215 (10), 85 (12), 77 (19), 63 (100). Analysis: calcd. for $C_{27}H_{19}N_{11}S_2$ (561.64): C, 57.74; H, 3.41; N, 27.43%; found: C, 57.69; H, 3.45; N, 27.45%.

**Reaction of the thiosemicarbazide 3a with α-halo compounds**

***General procedure***

To a solution of the thiosemicarbazide derivative **3a** (0.6 g, 1 mmol) and the appropriate α-halo compounds (phenacyl chloride, chloroacetone and ethyl chloroacetate) (2 mmol) in absolute EtOH (20 mL), a catalytic amount of triethylamine was added. The reaction mixture was refluxed for 4-7 h, and then allowed to cool. The precipitated solid was filtered off, washed with ethanol, dried, and finally recrystallized from EtOH to afford the corresponding thiazol-2-ylidene derivatives **7a**, **7b** and **9**, respectively. The synthesized compounds together with their physical and spectral data are listed below:

**2,6-Bis[4-(3,4-diphenylthiazol-2(3*H*)-ylideneaminocarbamoyl)-1*H*-pyrazole-3-yl]pyridine (7a)**

Yield (0.67 g, 84%); yellow crystals, m.p.: 230-232°C. IR (KBr, $cm^{-1}$): 3358-3210 (2NH, overlapped), 1687 (C=O). $^1H$ NMR ($CDCl_3$, δ, ppm): 6.44 (s, 2H, thiazole-H) 6.75-7.23 (m, 20H, Ar-H), 7.75-8.10 (m, 3H, pyridine-H), 8.15 (s, 2H, 2CH pyrazole), 8.43, 11.45 (br s, 2H, 2NH, $D_2O$-exchangeable). $^{13}C$ NMR ($CDCl_3$, δ, ppm): 103.4, 105.1, 114.6, 123.7, 123.6, 124.3, 126.7, 127.9, 128.9, 129.6, 130.4, 130.7, 138.6, 137.5, 140.1, 141.1, 147.1, 164.2. MS *m/z* (%): 797 [$M^+$] (1), 665 (5), 415 (7), 252 (44), 135 (85), 93 (17), 77 (100). Analysis: calcd. for $C_{43}H_{31}N_{11}O_2S_2$ (797.91): C, 64.73; H, 3.92; N, 19.31%; found: C, 64.61; H, 3.87; N, 19.36%.

**2,6-Bis[4-(4-methyl-3-phenylthiazol-2(3*H*)-ylideneaminocarbamoyl)-1*H*-pyrazol-3-yl]pyridine (7b)**

Yield (0.61 g, 91%); brown crystals; m.p.: 97-99°C. IR (KBr, $cm^{-1}$): 3439, 3181 (2NH, overlapped), 1673 (C=O). $^1H$ NMR ($CDCl_3$, δ, ppm): 2.23 (s, 6H, $2CH_3$), 6.25 (s, 2H, thiazole-H), 6.65-7.21 (m, 10H, Ar-H), 7.65-8.22 (m, 3H, pyridine, 2H, 2CH pyrazole), 8.57, 10.52 (br s, 2H, 2NH, $D_2O$-exchangeable). MS *m/z* (%): 673 [$M^+$] (2), 654 (8), 313 (18), 296 (13), 190 (7), 149 (100), 58 (91). Analysis: calcd. for $C_{33}H_{27}N_{11}O_2S_2$ (673.77): C, 58.83; H, 4.04; N, 22.87%; found: C, 58.76; H, 4.01; N, 22.79%.

**2,6-Bis[4-(4-oxo-3-phenylthiazolidin-2-ylideneaminocarbamoyl)-1*H*-pyrazol-3-yl]pyridine (9)**

Yield (0.55 g, 81%); yellow crystals; m.p.: 152-153°C. IR (KBr, $cm^{-1}$): 3193, 3141 (2NH), 1734, 1644 (2C=O). $^1H$ NMR ($CDCl_3$, δ, ppm): 4.23 (s, 4H, $2CH_2$), 6.98-7.23 (m, 10H, Ar-H), 7.55-8.02 (m, 3H, pyridine-H), 8.10 (s, 2H, 2CH, pyrazole), 8.55, 10.52 (br s, 2H, 2NH, $D_2O$-exchangeable). MS *m/z* (%): 677 [$M^+$] (5), 204 (8), 165 (10), 149 (31), 93 (7), 77 (23), 58 (100). Analysis: calcd. for $C_{31}H_{23}N_{11}O_4S_2$ (677.72): C, 54.94; H, 3.42; N, 22.73%; found: C, 54.88; H, 3.41; N, 22.76%.

**2,6-Bis[4-(3,5-dimethyl-1-carbonyl)-1*H*-pyrazol-3-yl]pyridine (10)**

To a mixture of the acid hydrazide **1** (0.33 g, 1 mmol) and acetylacetone (0.20 g, 2 mmol) in EtOH (10 mL), few drops of piperidine were added. The reaction mixture was refluxed for 6 h and then allowed to cool. The precipitated solid was filtered off, washed with water, dried and finally recrystallized from EtOH to afford compound **10**. Yield (0.39 g, 86%); buff powder (EtOH); m.p.: 137-138°C. IR (KBr, $cm^{-1}$): 3228 (NH), 1687 (C=O). $^1H$ NMR (DMSO-$d_6$, δ, ppm): 2.32 (s, 6H, $2CH_3$), 2.46 (s, 6H, $2CH_3$), 5.59 (s, 2H, pyrazole-H), 7.66-8.01 (m, 3H, pyridine-H), 8.13 (s, 2H, pyrazole-H), 12.95 (s, 2H, 2NH, $D_2O$-exchangeable). MS *m/z* (%): 455 [$M^+$] (2), 449 (9), 356 (30), 289 (14), 249 (14), 211 (16), 172 (40), 97 (100), 76 (41). Analysis: calcd. for $C_{23}H_{21}N_9O_2$ (455.47): C, 60.65; H, 4.65; N, 27.68%; found: C, 60.72; H, 4.58; N, 27.61%.

**2,6-Bis[4-(3-methylpyrazol-5-one-1-carbonyl)-1*H*-pyrazol-3-yl]pyridine (11)**

To a mixture of the acid hydrazide **1** (0.33 g, 1 mmol) and ethyl acetoacetate (0.26 g, 2 mmol) in EtOH (20 mL), a few drops of piperidine were added. The reaction mixture was refluxed for 5 h. The precipitated solid was filtered off, washed with water, dried and finally recrystallized from EtOH to afford compound **11**. Yield (0.32 g, 71%); brown powder (EtOH); m.p.: 163-164°C. IR (KBr, $cm^{-1}$): 3310 (NH), 1716, 1656 (C=O). $^1H$ NMR (DMSO-$d_6$,

δ, ppm): 2.32 (s, 6H, $2CH_3$), 4.21 (s, 2H, $2CH_2$), 5.79 (s, 2H, pyrazole-H), 7.86-8.11 (m, 3H, pyridine), 8.17 (s, 2H, pyrazole-H), 10.95 (s, 2H, 2NH, $D_2O$-exchangeable). MS *m*/*z* (%): 459 [$M^+$] (3), 431 (42), 358 (45), 284 (39), 180 (95), 76 (100). Analysis: calcd. for $C_{21}H_{17}N_9O_4$ (459.42): C, 54.90; H, 3.73; N, 27.44%; found: C, 55.02; H, 3.71; N, 27.52%.

**Condensation of acid hydrazide (1) with ketones**

To a solution of the acid hydrazide **1** (1 mmol) and the appropriate ketones (acetophenone or acetone) (2 mmol) in EtOH (10 mL), few drops of glacial acetic acid were added. The reaction mixture was refluxed for 5-9 h then allowed to cool to room temperature. The precipitated solid was filtered off, washed with ethanol, dried and finally recrystallized from DMF-EtOH to give the corresponding products **12a**,**b**, respectively. The synthesized compounds together with their physical and spectral data are listed below:

**Pyridine-2,6-bis[(*Z*)-*N*'-(1-methylethylidene)-3-yl-2*H*-pyrazole-4-carbohydrazide] (12a)**

Yield (0.39 g, 85%); white powder (EtOH), m.p.: 250-252°C. IR (KBr, $cm^{-1}$): 3231-3201 (2NH), 1645 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 2.40, 2.50 (2s, 12H, $4CH_3$), 7.89-8.33 (m, 3H, pyridine, 2H, pyrazole-H), 10.21 (s, 2H, 2NH, $D_2O$-exchangeable), 12.51 (s, 2H, 2NH, $D_2O$ exchangeable). MS *m*/*z* (%):407 [$M^+$] (11). Analysis: calcd. for $C_{19}H_{21}N_9O_2$ (407.43): C, 56.01; H, 5.20; N, 30.94%; found: C, 56.11; H, 5.26; N, 30.87%.

**Pyridine-2,6-bis[(*Z*)-*N*'-(1-phenylethylidene)-3-yl-2*H*-pyrazole-4-carbohydrazide] (12b)**

Yield (0.41 g, 77%); white powder (EtOH); m.p.: 245-247°C. IR (KBr, $cm^{-1}$): 3224 (NH), 1667 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 2.10 (s, 6H, $2CH_3$), 7.36-7.71 (m, 10H Ar-H), 7.75-8.35 (m, 3H, pyridine and pyrazole CH-protons), 10.53 (br s, 2H, 2NH, $D_2O$-exchangeable), 12.46 (br s, 2H, 2NH, $D_2O$-exchangeable). $^{13}$C NMR (DMSO-$d_6$, δ, ppm): 15.2, 111.1, 121.2, 126.6, 127.7, 128.8, 128.9, 129.1, 138.3, 149.1, 157.8, 159.9, 163.5. MS *m*/*z* (%): 531 [$M^+$] (2), 464 (5), 398 (69), 297 (11), 238 (72), 171 (33), 118 (47), 77 (100). Analysis: calcd. for $C_{29}H_{25}N_9O_2$ (531.57): C, 65.53; H, 4.74; N, 23.71%; found: C, 65.61; H, 4.69; N, 23.67.

**Condensation of the acid hydrazide (1) with aromatic aldehydes**

To a mixture of the acid hydrazide **1** (0.33 g, 1 mmol) and the appropriate aldehyde (2 mmol), in EtOH (10 mL), few drops of glacial acetic acid were added. The reaction mixture was refluxed for 4-7 h then left to cool. The precipitated product was filtered off, washed with EtOH, dried and finally recrystallized from the appropriate solvent to afford the corresponding Schiff bases **13a-d**.

**Pyridine-2,6-bis[(*N*'-benzylidene-2*H*-pyrazol-3-yl)-4-carbohydrazide] (13a)**

Yield (0.35 g, 69%); white powder (EtOH), m.p.: 266-268°C. IR (KBr, $cm^{-1}$): 3201 (NH), 1687 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 7.10-7.21 (m, 10H, Ar-H), 7.63-8.18 (m, 3H, pyridine-H, 2H, pyrazole-H, 2CH, imine-CH), 10.85 (br s, 2H, 2NH, $D_2O$-exchangeable), 12.41 (br s, 2H, 2NH, $D_2O$-exchangeable). MS *m*/*z* (%): 503 [$M^+$] (14), 352 (9), 290 (11), 230 (15), 188 (91), 171 (100), 89 (67), 77 (42). Analysis: calcd. for $C_{27}H_{21}N_9O_2$ (503.51): C, 64.41; H, 4.20; N, 25.04%; found: C, 64.49; H, 4.27; N, 25.11%.

**Pyridine-2,6-bis[(*N*'-4-methoxybenzylidene-2*H*-pyrazol-3-yl)-4-carbohydrazide] (13b)**

Yield (0.41 g, 73%); white powder (EtOH); m.p.: 235-237°C. IR (KBr, $cm^{-1}$): 3260-3116 (2NH), 1663 (C=O). $^1$H NMR (DMSO-$d_6$, δ, ppm): 3.73 (s, 6H, $2CH_3$), 7.05-7.25 (m, 8H, Ar-H), 7.79-8.10 (m, 3H, pyridine-H, 2CH, imine-CH), 8.21 (s, 2H, pyrazole-H), 11.71 (br s, 2H, 2NH, $D_2O$-exchangeable), 12.41 (br s, 2H, 2NH, $D_2O$-exchangeable). MS *m*/*z* (%):563 [$M^+$] (5), 379 (20), 262 (17), 230 (30), 171 (100), 116 (61), 77 (37). Analysis: calcd. for $C_{29}H_{25}N_9O_4$ (563.57): C, 61.80; H, 4.47; N, 22.37%; found: C, 61.71; H, 4.41; N, 22.43%.

**Pyridine-2,6-bis[(*N*'-4-methylbenzylidene-2*H*-pyrazol-3-yl)-4-carbohydrazide] (13c)**

Yield (0.43 g, 81%); white powder (EtOH); m.p.: 270-272°C. IR (KBr, $cm^{-1}$): 3176, 3106 (2NH), 1647 (C=O).$^1$H NMR (DMSO-$d_6$, δ, ppm): 2.60, 2.69 (s, 6H, $2CH_3$), 7.17-7.31 (m, 8H, Ar-H), 7.63-8.05 (m, 3H, pyridine-H, 2CH, imine-CH), 8.13 (s, 2H, pyrazole-H), 11.76 (br s, 2H, 2NH, $D_2O$-exchangeable), 12.42 (br s, 2H, 2NH, $D_2O$-exchangeable). MS *m*/*z* (%): 531 [$M^+$] (2), 505 (10), 92 (100), 77 (42). Analysis: calcd. for $C_{29}H_{25}N_9O_2$ (531.57): C, 65.53; H, 4.74; N, 23.71%; found: C, 65.57; H, 4.72; N, 23.79%.

***In vitro* assay for anti-cancer activity**

The synthesized compounds were supplied to the Bioassay-Cell Culture Laboratory, National Research Centre, Cairo, Egypt for *in vitro* primary antitumor screening on lung adenocarcinoma

(A549), hepatocellular carcinoma (HEPG2) and caucasian breast adenocarcinoma (MCF7) (American Type Culture Collection). Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan (21, 22).

**Procedure**

The following procedures were done in a sterile area using a laminar flow cabinet biosafety class II level (Baker, SG403INT, Sanford, ME, USA). HEPG-2 cell line was cultured in RPMI-1640 and MCF-7 and A549 cell line were cultured in DMEM. Cells were applied in 96-well plates (having about 10000 cells /well). The plates were then incubated for 24 h in 37$^{o}$C incubation and 5% $CO_2$ atmosphere before treatment with the tested compounds to allow attachment of cells to the wall of the plate. The tested compounds were dissolved in DMSO. Different concentrations of the compounds under test were added to the cell monolayer. Triplicate wells were prepared for each individual concentration, and then the plate was incubated for 48 h in 37$^{o}$C incubator. Forty microliters of MTT solution (2.5 mg/mL) was added into each well for additional 4 h. Formazan was dissolved in 200 µL (10%) sodium dodecyl sulfate and then measured at absorbance at $\lambda = 495$ nm. Cell viability at given compound concentration was calculated as the percentage of absorbance in wells with the compound-treated cells to that of vehicle control cells (100%). The active compounds that gave > 75% at 100 µM, were submitted to calculate their $IC_{50}$ values (the concentration that inhibited cell viability by 50%) (22).

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## NATURAL DRUGS

# PHYTOCHEMISTRY, ANTIOXIDATIVE AND ANTIDIABETIC EFFECTS OF VARIOUS PARTS OF *EUGENIA CARYOPHYLLATA* THUNB. *IN VITRO*

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**Abstract:** *Eugenia caryophyllata* Thunb. possesses a wide variety of therapeutic potential and has been recognized as a source of antioxidative and antidiabetic agents. This study was designed to investigate and compare the antioxidative and antidiabetic effects of different parts (bud, leaf, stem and root) of *E. caryophyllata*. Samples were sequentially extracted using solvents of increasing polarity and investigated for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, total reducing power, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase activities as markers of *in vitro* antidiabetic effects. Phytochemicals were analyzed using gas chromatography-mass spectrometry (GC-MS) analysis. The ethanol (EtOH) extracts of the bud, leaf and stem exhibited a higher total polyphenol and flavonoid content compared to other extracts, with the EtOH extracts of the bud and leaf exhibiting lower $IC_{50}$ values than the extracts of the other plant parts for all the models used in this study (DPPH bud: 0.17 ± 0.20 mg/mL; leaf: 0.03 ± 0.01 mg/mL; hemoglobin glycosylation: bud: 0.17 ± 0.02 mg/mL; 0.83 ± 0.04 mg/mL; α-amylase: bud: 0.20 ± 0.02 mg/mL; 322.27 ± 73.29 mg/mL; α-glucosidase: bud: 0.03 ± 0.01 mg/mL; 0.74 ± 0.02 mg/mL). A similar result was observed for the reducing potentials of $Fe^{3+}$ to $Fe^{2+}$ by the extract. The GC-MS analysis of these parts indicated several aromatic phenols, acids, carophyllene and long chain aliphatic acids.Conclusively, various solvent extracts from the leaf, bud and stem of *E. caryophyllata* showed higher antioxidative and antidiabetic effects in comparison to common standards used in these assays.

**Keywords:** α-amylase, α-glucosidase, antidiabetic, antioxidative, *Eugenia caryophyllata*

With the increasing cost of drugs and the accessibility of these drugs to African countries, especially in rural and remote areas, the popularity of using medicinal plants for therapeutic purposes has increased significantly during the past decade. In Africa, about 80% of the population depend almost entirely on traditional medicine or herbal medicine, for their primary health care needs (1, 2). This is not surprising as several modern conventional drugs were originally obtained from plant sources and caused minimal or no side effects compared to synthetic drugs. For instance, metformin, an antidiabetic drug originated from *Galega officinalis* (3), quinine and quinidine, antiarrhythmic drugs were phytochemicals from *Cinchona spp* (4). In addition, the perceived effectiveness of the herbal therapies as well as the availability of these medicinal plants makes them a popular source of medicines. Moreover, the African continent accounts for about 25% of the total number of higher plants in the world, where more than 5400 medicinal plants are reported to have over 16300 medicinal uses (5). Consequently, the World Health Organization (WHO) has encouraged researchers to investigate and validate the folklore uses of plants used in the treatment of various diseases such as diabetes mellitus, hypertension, malaria and microbial infections among others (6).

*Eugenia caryophyllata* Thunb. (Syn. *Syzygium aromaticum* (Linn.) Merr. & L.M. Perry) or clove is an aromatic plant that belongs to the *Myrtaceae* family (7) and is widely available in Africa, Asia and North America. The bud and leaf are locally used as spice in various food preparations and possess a wide variety of therapeutic potential (8). *E. caryophyllata* parts or extracted oils are traditionally used in the treatment of toothache (9) and also been reported to have a strong antimicrobial effect (10). It is locally utilized in the treatment of asthma in Asia (11), disorders associated with respiratory

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and digestive systems (12) and in diarrheal and sexual disorders (13).

Previous studies reported that *E. caryophyllata* bud and its essential oils possessed strong antioxidative properties and thus terminate lipid peroxidation and other oxidative processes (14, 15). Adefegha and Oboh (16) reported the ability of the bud to inhibit α-amylase and α-glucosidase activities *in vitro*. The insulinotropic effect (17) and hepatoprotective effect against ethanol-induced liver cell injury (18) of the bud has also been reported. More recently, antihyperglycemic, hypolipidemic, hepatoprotective and antioxidative effects of clove powder have also been reported (19). Active principles identified in the bud or its essential oils include eugenol, β-caryophyllene, fatty acids, triterpenes, alcohols, flavonoids and other phenolics (20). It is hypothesized that since *E. caryophyllata* buds have been reported to have antioxidative and antidiabetic effects, other parts of the plant may also have similar activities and possess similar bioactive compounds. However, till now, the majority of scientific investigations had only focused on the buds and its essential oils. No data is available on the validity or potential of extracts of the other parts of the plant. Thus, this study was designed to investigate and compare the antioxidative and antidiabetic effects of various parts (including bud) of *E. carophyllata* using several *in vitro* models. Additionally, phytochemical analysis of the possible bioactive compounds present in most active extracts was also carried out using GC-MS analysis.

## MATERIALS AND METHODS

### Chemicals and reagents

Ascorbic acid, quercetin, hemoglobin (human lyophilized powder), gallic acid, aluminum chloride, α-amylase from porcine pancreas, α-glucosidase from *Saccharomyces cerevisiae* and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were purchased from Sigma-Aldrich through Capital Lab Supplies, New Germany, South Africa. Folin Ciocalteau reagent was purchased from Merck Chemical Company, South Africa. Gentamycin was purchased from EMD Chemicals, San Diego, CA, USA.

### Plant material

The bud, leaf, stem and root samples of *E. carophyllata* were identified and authenticated at the herbarium unit of the Biological Science Department, Ahmadu Bello University, Zaria, Nigeria by Mr. Umar Gallah and a voucher specimen number 13209 was deposited accordingly. The plant samples were immediately washed and shade-dried to constant weights for two weeks. The dried samples were ground to a fine powder, and then stored individually in airtight containers to transport to the University of KwaZulu-Natal, Westville campus, Durban, South Africa for subsequent analysis.

### Preparation of the plant extracts

Forty (40) grams of each of the fine powdered plant parts were separately defatted with 200 mL of n-hexane. The defatted material was sequentially extracted with ethyl acetate, ethanol and water by soaking for 48 h in 200 mL of the relevant solvent followed by a 2 h orbital shaking at 200 rpm. After filtration through Whatmann filter paper (No. 1), respective solvents were evaporated under vacuum, using a rotary evaporator (Buchi Rotavapor II, Buchi, Germany) at 40$^{o}$C under reduced pressure to obtain the different solvent extracts with the exception of the aqueous extracts which were dried on a water bath at 45$^{o}$C. The extracts in each case were weighed, transferred to micro tubes and stored in a refrigerator at 4$^{o}$C until further analysis.

### Estimation of total polyphenol content

The total polyphenol content of each extract was determined (as gallic acid equivalent) according to the method described by McDonald et al. (21) with slight modifications. Briefly, 200 µL of the extract (240 µg/mL) was incubated with 1 mL of 10 times diluted Folin Ciocalteau reagent and 800 µL of 0.7 M $Na_2CO_3$ for 30 min at room temperature. The absorbance values were then determined at 765 nm in a Shimadzu UV mini 1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). All measurements were done in triplicate.

### Determination of total flavonoid content

The total flavonoid content of the plant extracts were determined using a method reported by Chang et al. (22) with slight modification. Briefly, 500 µL (240 µg/mL) of each sample was mixed with 500 µL methanol, 50 µL of 10% $AlCl_3$, 50 µL of 1 mol/L potassium acetate and 1.4 mL water, and allowed to incubate at room temperature for 30 min. The absorbance of the reaction mixture was subsequently measured at 415 nm using the spectrophotometer mentioned above. The total flavonoid content was calculated as quercetin equivalent (QE) in µg per mg dry extract.

### DPPH radical scavenging activity

The total free radical scavenging activity of the extracts was determined and compared to that of

ascorbic and gallic acids by using a slightly modified method described by Tuba & Gulcin (23). An aliquot of 500 µL of a 0.3 mM solution of DPPH in methanol was added to 1 mL of the extracts at different concentrations (30, 60, 120 and 240 µg/mL). These solutions were mixed and incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against blank samples lacking the free radical scavengers.

**Ferric ($Fe^{3+}$) reducing antioxidant power assay**

The ferric reducing antioxidant power method of Oyaizu (24) was used with slight modifications to measure the reducing capacity of the extracts. To perform this assay, 1 mL of each extract (30, 60, 120 and 240 µg/mL) was incubated with 1 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 1% potassium ferricyanide at 50°C for 30 min. After 30 min incubation, the reaction mixtures were acidified with 1 mL of 10% trichloroacetic acid. Thereafter, 1 mL of the acidified sample of this solution was mixed with 1 mL of distilled water and 200 µL of $FeCl_3$ (0.1%). The absorbance of the resulting solution was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicated greater reductive capability of the extracts (15).

**Inhibition of hemoglobin glycosylation**

Inhibition of non-enzymatic glycosylation of hemoglobin by various extracts was investigated by the modified method of Pal & Dutta (25). Glucose (2%), hemoglobin (0.06%) and gentamycin (0.02%) solutions were prepared in phosphate buffer 0.01 M, pH 7.4. An aliquot of 1 mL of each of the solutions were mixed with 1 mL of different concentration of the extracts (30, 60, 120 and 240 µg/mL) in dimethyl sulfoxide (DMSO). These mixtures were incubated in the dark at room temperature for 72 h. The percentage inhibition of glycosylation of hemoglobin was calculated from the absorbance measured at 520 nm. Gallic acid was used as a standard.

**α-Amylase inhibitory effect**

The α-amylase inhibitory effect of the extracts was carried out using a modified method of McCue and Shetty (26). Briefly, a 250 µL aliquot of extract at different concentrations (30, 60, 120 and 240 µg/mL) was placed in a tube and 250 µL of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution was added. This solution was preincubated at 25°C for 10 min, after which 250 µL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at a time interval of 10 s and then further incubated at 25°C for 10 min. The

Table 1. Percentage yield, total polyphenol and flavonoid contents of various solvent extracts of *E. caryophyllata* parts.

| Samples | Yield (%) | Total polyphenols content (mg/g GAE) | Total flavonoids content (mg/g QE) |
|---|---|---|---|
| **Bud** | | | |
| Ethyl acetate | 2.75 | $9.68 \pm 0.26^{e}$ | $1.08 \pm 0.29^{a}$ |
| Ethanol | 5.00 | $27.23 \pm 1.45^{h}$ | $10.64 \pm 0.59^{e}$ |
| Aqueous | 1.25 | $11.17 \pm 0.11^{f}$ | $3.19 \pm 0.41^{c}$ |
| **Leaf** | | | |
| Ethyl acetate | 0.85 | $7.45 \pm 0.11^{d}$ | $1.11 \pm 0.24^{a}$ |
| Ethanol | 4.05 | $13.83 \pm 0.22^{g}$ | $5.72 \pm 1.59^{d}$ |
| Aqueous | 1.55 | $6.94 \pm 0.10^{d}$ | $2.17 \pm 0.53^{b}$ |
| **Root** | | | |
| Ethyl acetate | 0.17 | $2.07 \pm 0.08^{c}$ | $0.72 \pm 0.18^{a}$ |
| Ethanol | 0.75 | $2.09 \pm 0.05^{c}$ | $1.64 \pm 0.35^{b}$ |
| Aqueous | 0.55 | $2.65 \pm 0.05^{c}$ | $0.58 \pm 0.12^{a}$ |
| **Stem** | | | |
| Ethyl acetate | 0.62 | $0.66 \pm 0.05^{a}$ | $0.17 \pm 0.12^{a}$ |
| Ethanol | 2.77 | $6.12 \pm 0.11^{d}$ | $2.47 \pm 0.12^{b}$ |
| Aqueous | 1.45 | $1.40 \pm 0.12^{b}$ | $0.33 \pm 0.12^{a}$ |

Data are presented as the mean ± SD values of triplicate determinations. $^{a-h}$Different superscripted letters within a column are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$).

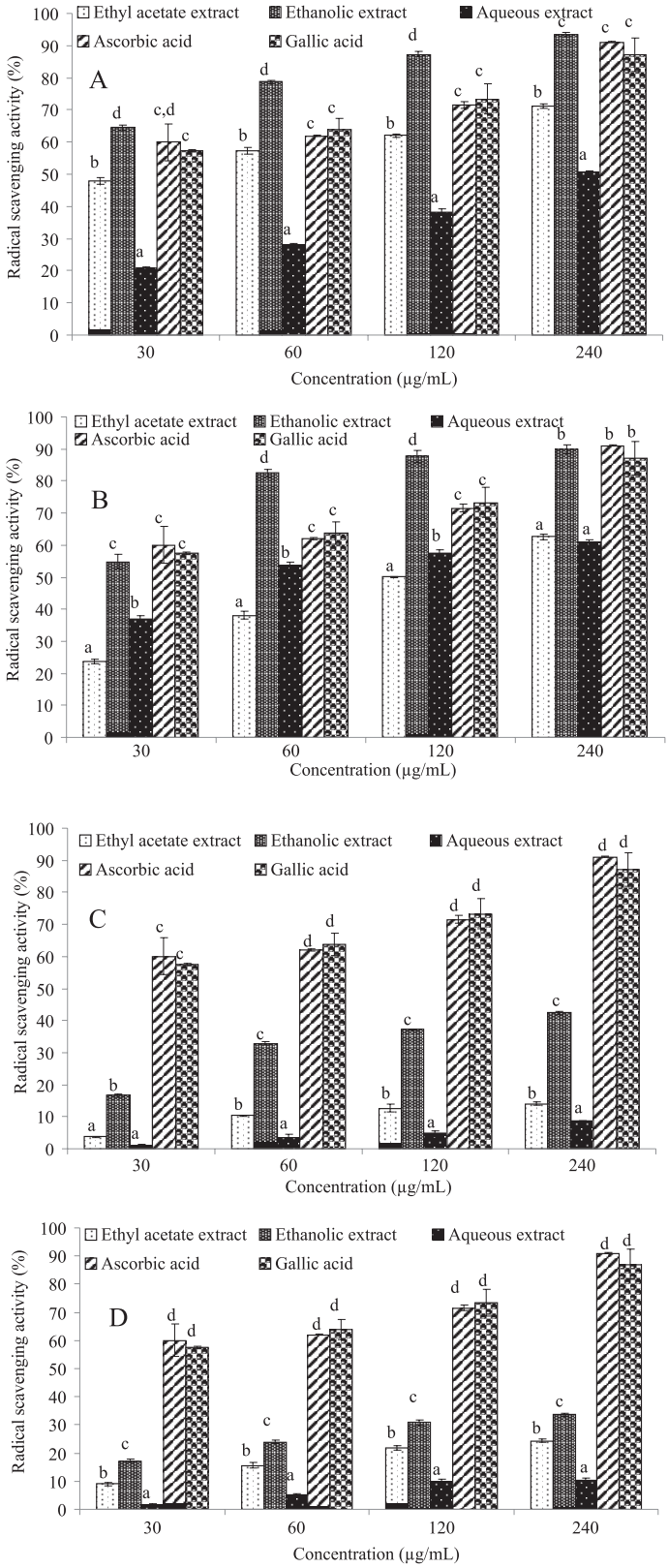


Figure 1. DPPH radical scavenging activity (%) of bud (A), leaf (B), root (C) and stem (D) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. [a-d] Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range *post hoc* test, $p < 0.05$).

reaction was terminated after incubation by adding 1 mL of dinitrosalicylic acid (DNS) reagent. The tube was then boiled for 10 min and cooled to room temperature. The reaction mixture was diluted with 5 mL of distilled water and the absorbance was measured at 540 nm using a Shimadzu UV mini 1240 spectrophotometer. A control was prepared using the same procedure, replacing the extract with distilled water.

**α-Glucosidase inhibitory effect**

The inhibitory effect of the plant extracts on α-glucosidase activity was determined according to the method described by Kim et al. (27) using α-glucosidase from *Saccharomyces cerevisiae*. The substrate solution p-nitrophenyl glucopyranoside (pNPG) was prepared in 20 mM phosphate buffer, pH 6.9. An aliquot of 500 µL of α-glucosidase was then preincubated with 250 µL of the different concentrations of the extracts (30, 60, 120 and 240 µg/mL) for 10 min. Thereafter, 250 µL of 5.0 mM pNPG was dissolved in 20 mM phosphate buffer (pH 6.9) as a substrate to start the reaction. The reaction mixture was incubated at 37°C for 30 min. The α-glucosidase activity was determined by measuring the yellow colored p-nitrophenol released from pNPG at 405 nm. The results of the DPPH, inhibition of hemoglobin glycosylation, α-amylase and α-glucosidase assays were expressed as a percentage of the control (blank) according to the following formula:

$$\% \text{ Inhibition} = [(\text{Abs. of control} - \text{Abs. of extract})/\text{Abs. of control}] \times 100$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity ($IC_{50}$) were calculated from the data as well.

**Gas chromatography-mass spectroscopic (GC-MS) analysis**

Based on the results of antioxidative and antidiabetic studies, the most active extracts (EtOH bud, leaf and stem) were subjected to GC-MS analysis. The GC-MS analysis was conducted with an Agilent Technology 6890 gas chromatograph cou-

Table 2. $IC_{50}$ values of various solvent extracts of *E. caryophyllata* parts in different antioxidative and antidiabetic models.

| Samples | $IC_{50}$ (mg/mL) | | | |
|---|---|---|---|---|
| | DPPH scavenging activity | Non-enzymatic glycosylation of hemoglobin | α-Amylase inhibitory effect | α-Glucosidase inhibitory effect |
| **Bud** | | | | |
| Ethyl acetate | 0.06 ± 0.01[a] | 535.62 ± 372.42[b] | 320.36 ± 167.03[c] | 0.68 ± 0.03[a] |
| Ethanol | 0.02 ± 0.01[a] | 0.17 ± 0.02[a] | 0.20 ± 0.02[a] | 0.03 ± 0.01[a] |
| Aqueous | 0.62 ± 0.14[b] | 559.96 ± 377.16[b] | 386.36 ± 97.28[c] | 5260.41 ± 54.99[f] |
| **Leaf** | | | | |
| Ethyl acetate | 0.16 ± 0.03[a] | 301.92 ± 195.09[b] | 505.79 ± 32.95[d] | 414.04 ± 75.44[d] |
| Ethanol | 0.03 ± 0.01[a] | 0.83 ± 0.04[a] | 322.27 ± 73.29[c] | 0.74 ± 0.02[b] |
| Aqueous | 0.07 ± 0.02[a] | 594.56 ± 234.39[b] | 983.49 ± 62.64[e] | 596.30 ± 7.32[e] |
| **Root** | | | | |
| Ethyl acetate | 296.73 ± 117.28[d] | ND | ND | ND |
| Ethanol | 3.66 ± 1.16[c] | ND | ND | ND |
| Aqueous | 222980.33 ± 815.45[e] | ND | ND | ND |
| **Stem** | | | | |
| Ethyl acetate | 230.68 ± 99.66[d] | 1019.48 ± 75.97[c] | 1110.51 ± 108.53[e] | 89149.67 ± 107.99[h] |
| Ethanol | 0.99 ± 0.32[b] | 1.03 ± 0.42[a] | 349.53 ± 282.83[c] | 6.89±0.11[c] |
| Aqueous | 527647.61 ± 684.74[f] | 650.06 ± 276.14[b] | 2561.78 ± 109.69[f] | 83528.18 ± 1001.29[g] |
| Ascorbic acid | 0.03 ± 0.02[a] | ND | ND | ND |
| Gallic acid | 0.05 ± 0.01[a] | 0.20 ± 0.01[a] | ND | ND |
| Acarbose | ND | ND | 4.91 ± 0.80[b] | 0.34 ± 0.02[b] |

Data are presented as the mean ± SD values of triplicate determinations. [a-h] Different superscript letters presented within a column for a given parameter are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$). ND = Not determined.

pled with an Agilent 5973 Mass Selective Detector and driven by Agilent Chemstation software. Compounds were identified by direct comparison of the retention times and mass spectral data with those in the National Institute of Standards and Technology (NIST) library.

**Statistical analysis**

All data are presented as the mean ± SD of triplicates determination. Data were analyzed by using a statistical software package (SPSS for Windows, version 18, IBM Corporation, NY, USA) using Tukey's HSD multiple range *post-hoc* test. Values were considered significantly different at $p < 0.05$.

## RESULTS

The yield recovered from different solvent extracts of various parts of *E. carophyllata* indicated that higher yields are obtained from the bud and leaf extracts compared to roots and stem (Table 1). Furthermore, different parts showed variable amounts of polyphenols and flavonoid contents. EtOH extracts of various parts of the plant possessed a significantly ($p < 0.05$) higher total polyphenol and flavonoid content with the bud, leaf and stem containing the highest (Table 1). It was also observed that the aqueous extracts from the bud and stem showed a higher polyphenolic and flavonoid content compared to the ethyl acetate extracts. In the leaf, the aqueous extract had lower polyphenolic content than the ethyl acetate extract and in the root, the aqueous extract exhibited a lower flavonoid content compared to the ethyl acetate extract.

The ability of various solvent extracts to scavenge the DPPH radical were investigated and compared with ascorbic acid and gallic acid. The results are presented in Figure 1. It is evident from the results that the EtOH extracts from various parts of *E. caryophyllata* exhibited lower $IC_{50}$ values compared to other solvent extracts. The bud (A) and leaf (B) EtOH extracts demonstrated significantly ($p < 0.05$) lower $IC_{50}$ values of 0.02 ± 0.01 mg/mL and 0.03 ± 0.01 mg/mL, respectively, compared to other

Table 3. Phytochemicals identified in the EtOH extracts of the bud, leaf and stem by GC-MS

| Extracts | Compounds | Retention time (min) | Mass [a.m.u.] |
|---|---|---|---|
| **Bud** | | | |
| **1** | Eugenol | 6.67 | 164 $[M]^+$ |
| **3** | Caryophyllene | 7.21 | 204 $[M]^+$ |
| **4** | 2-Acetyl-4(2-propenyl)anisole | 7.72 | 206 $[M]^+$ |
| **5** | 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester | 11.33, 13.16 | 279 $[M + H]^+$ |
| **6** | n-Hexadecanoic acid | 10.04-10.22 | 256 $[M]^+$ |
| **7** | 9,12-Octadecadienal | 11.12, 14.40 | 264 $[M]^+$ |
| **8** | n-Octadecanoic acid | 11.21 | 284 $[M]^+$ |
| **9** | 9,12-Octadecadienoic acid | 11.50 | 280 $[M]^+$ |
| **10** | Heneicosanoic acid | 11.96 | 326 $[M]^+$ |
| **Leaf** | | | |
| **1** | Eugenol | 6.67 | 164 $[M]^+$ |
| **3** | Caryophyllene | 7.22 | 204 $[M]^+$ |
| **4** | 2-Acetyl-4(2-propenyl)anisole | 7.72 | 206 $[M]^+$ |
| **5** | 1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl) ester | 13.12 | 279 $[M + H]^+$ |
| **Stem** | | | |
| **1** | Eugenol | 6.66[a] | 164 $[M]^+$ |
| **2** | 2-Methoxy-3-(2-propenyl)phenol | 6.66[a] | 164 $[M]^+$ |
| **3** | Caryophyllene | 7.72 | 204 $[M]^+$ |

[a] isomers co-eluted at the same retention time

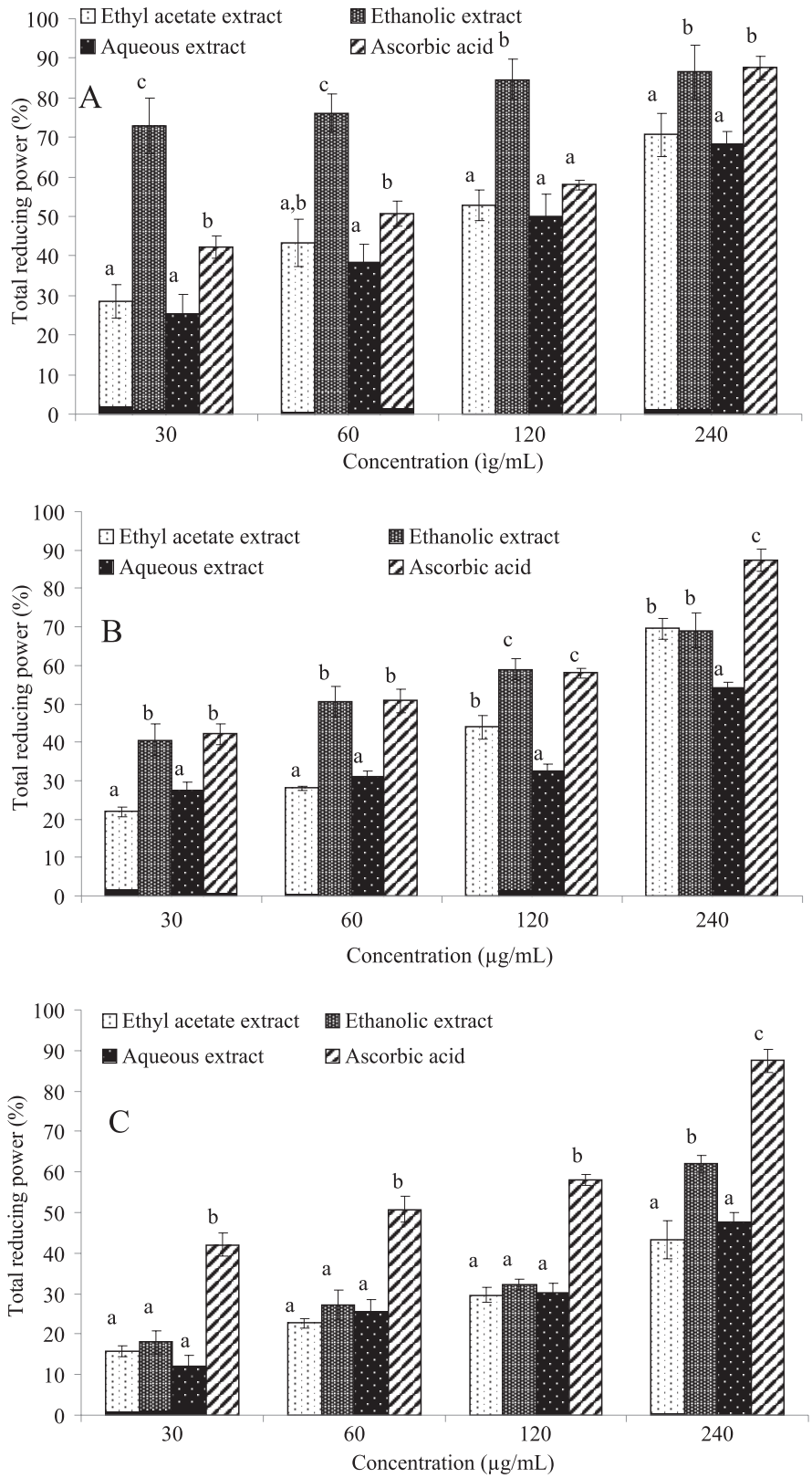


Figure 2. Total reducing power (relative to gallic acid) of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. [a-c] Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's-HSD multiple range *post hoc* test, $p < 0.05$)

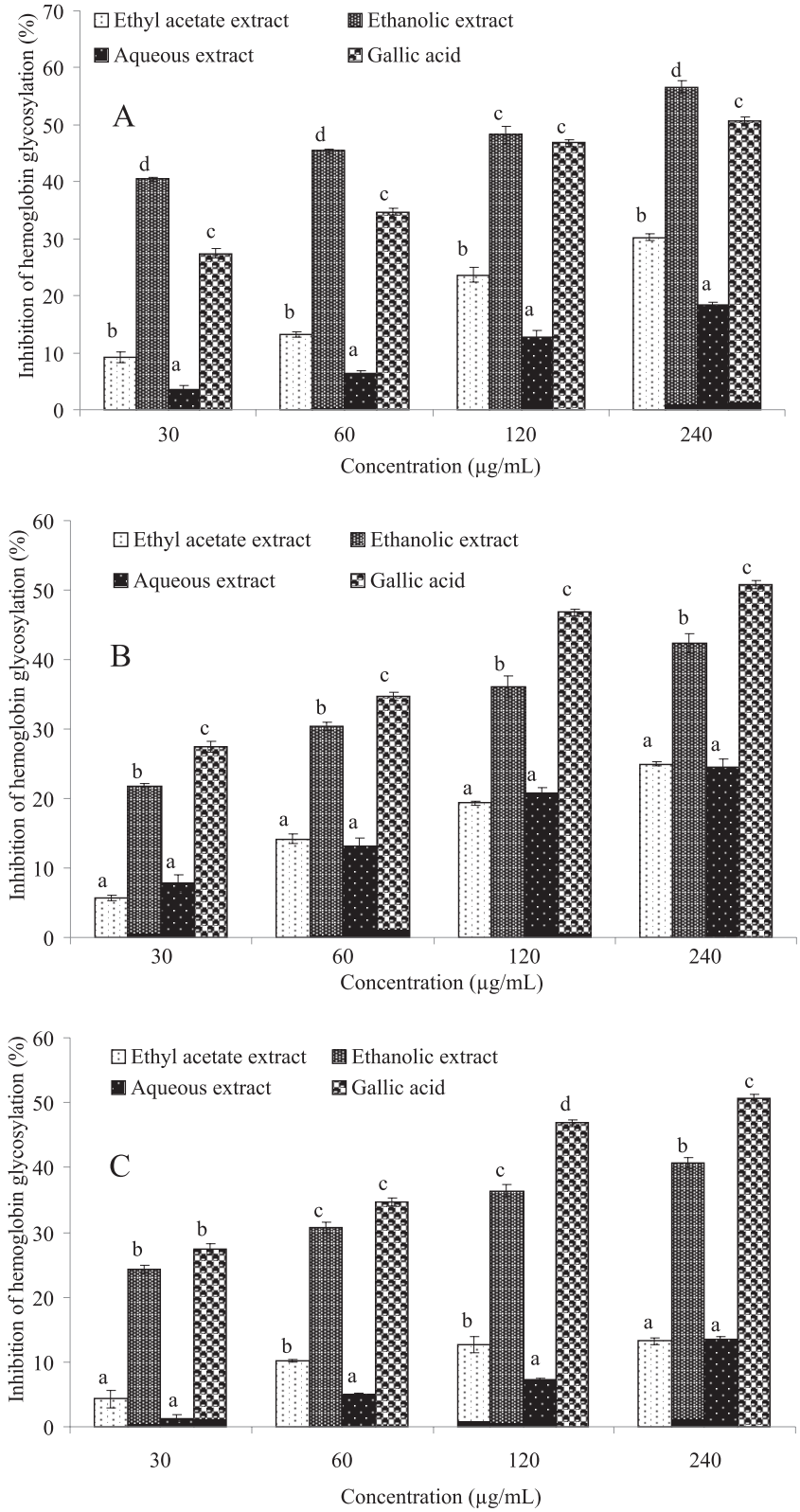


Figure 3. Inhibition of hemoglobin glycosylation (%) of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. [a-d] Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range *post hoc* test, $p < 0.05$)0

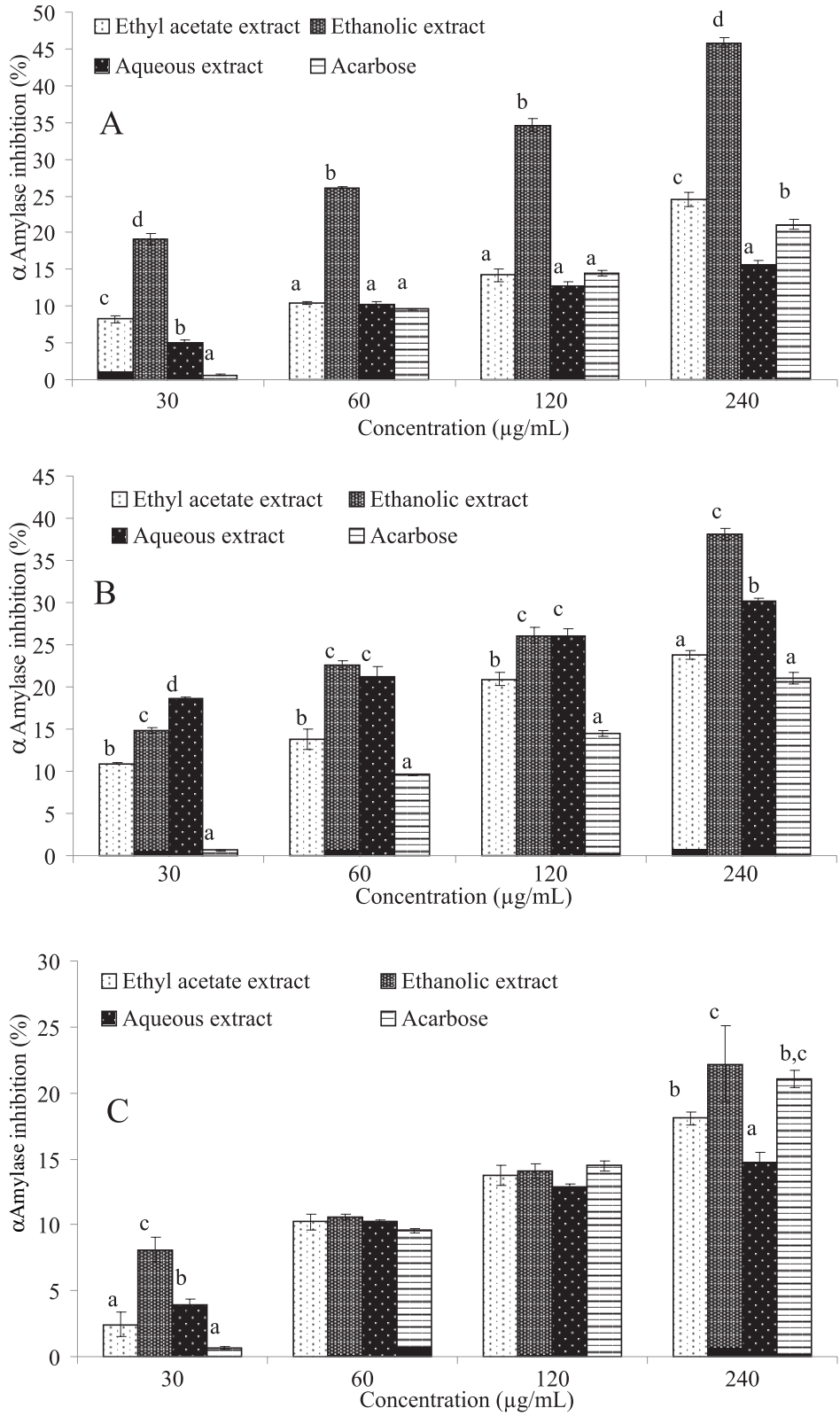


Figure 4. α-Amylase inhibition (%) of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. Data are presented as the mean ± SD of triplicate determinations. [a-d] Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range post hoc test, $p < 0.05$)

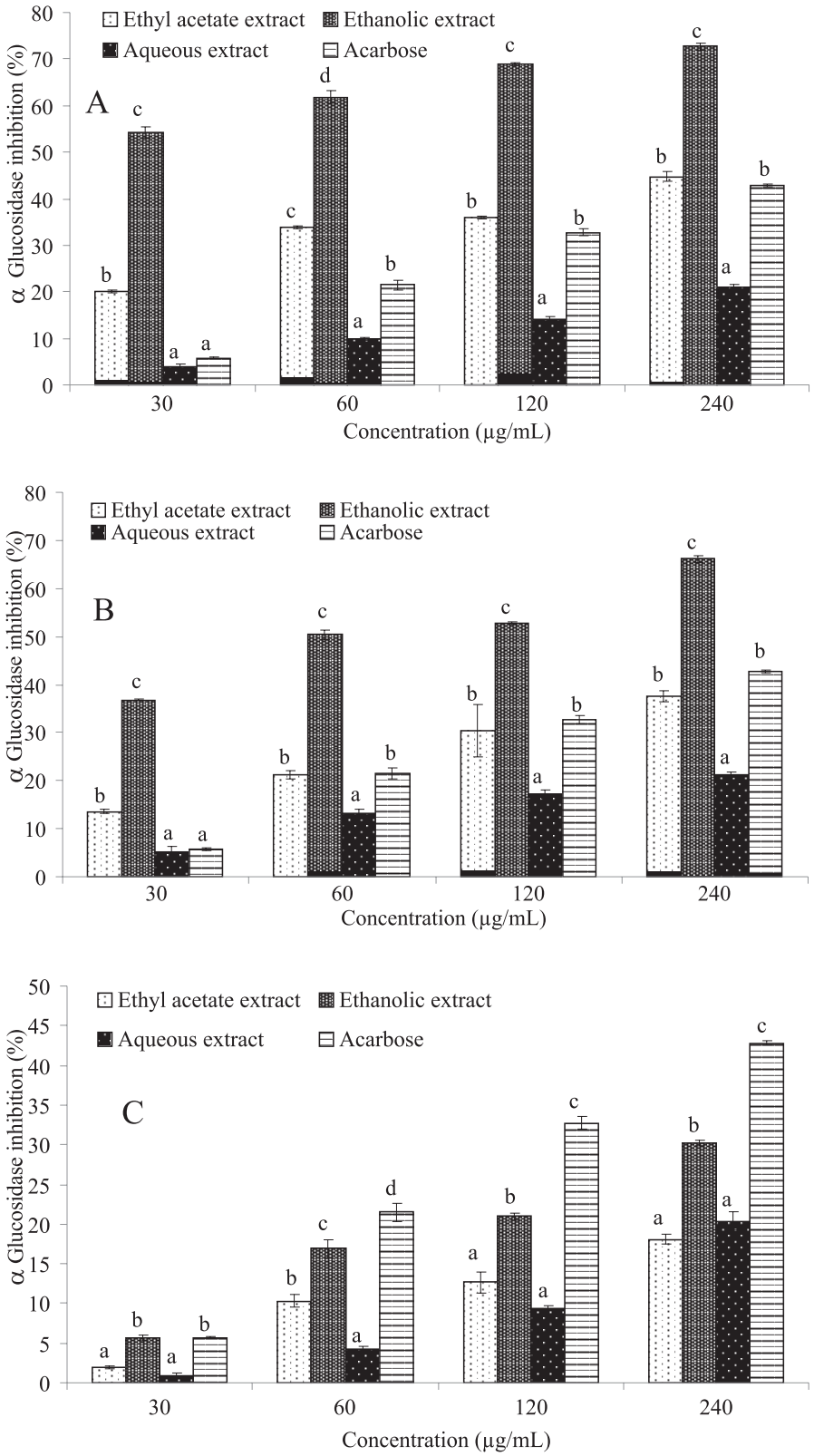


Figure 5. α-Glucosidase inhibition (%) of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*. Data are presented as mean ± SD of triplicate determinations. [a-d] Different letters presented over the bars for a given concentration of each extract are significantly different from each other (Tukey's HSD multiple range *post hoc* test, $p < 0.05$)

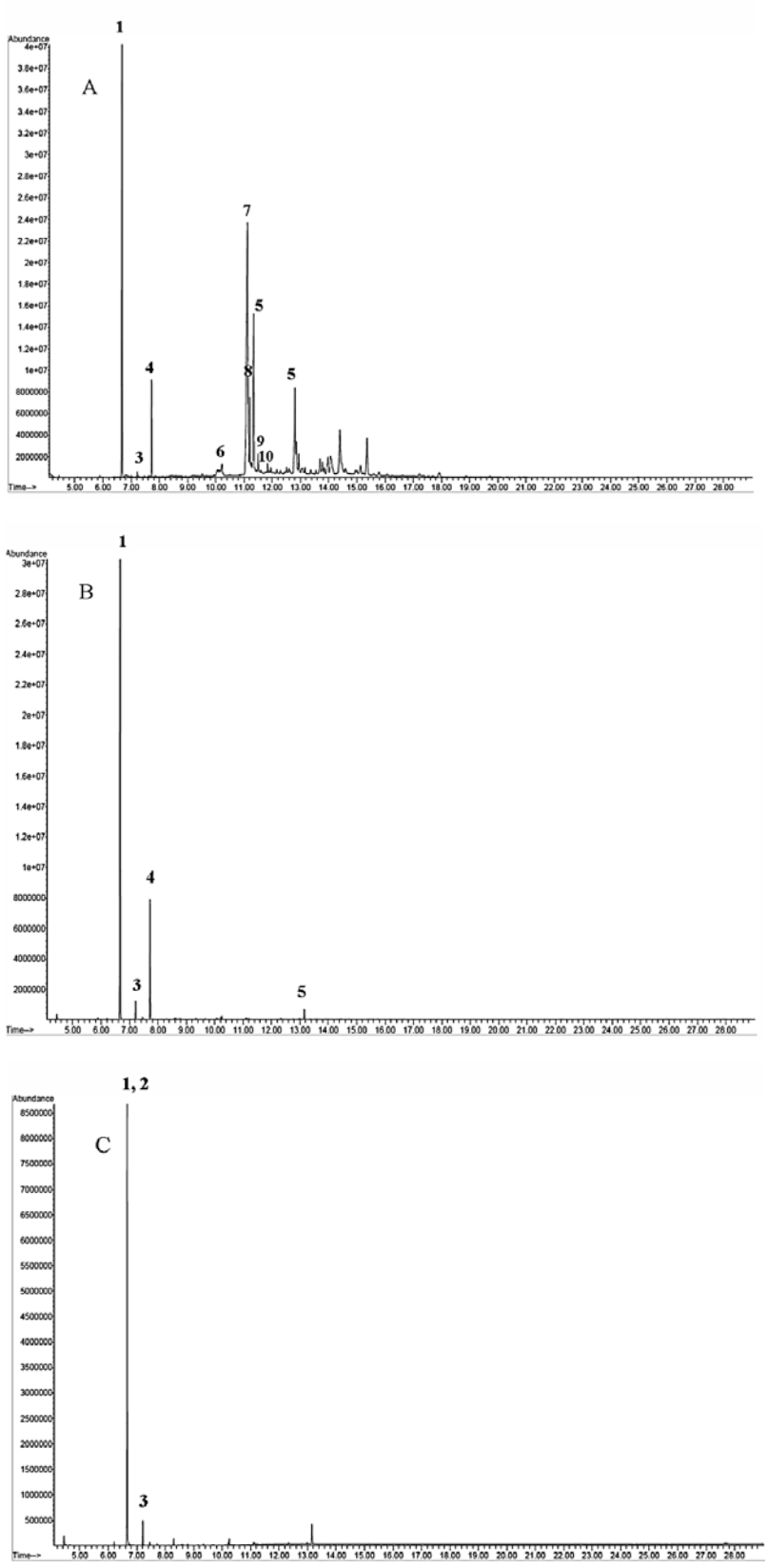


Figure 6. GC-MS chromatograms of ethanolic extracts of bud (A), leaf (B) and stem (C) extracts of *E. caryophyllata*

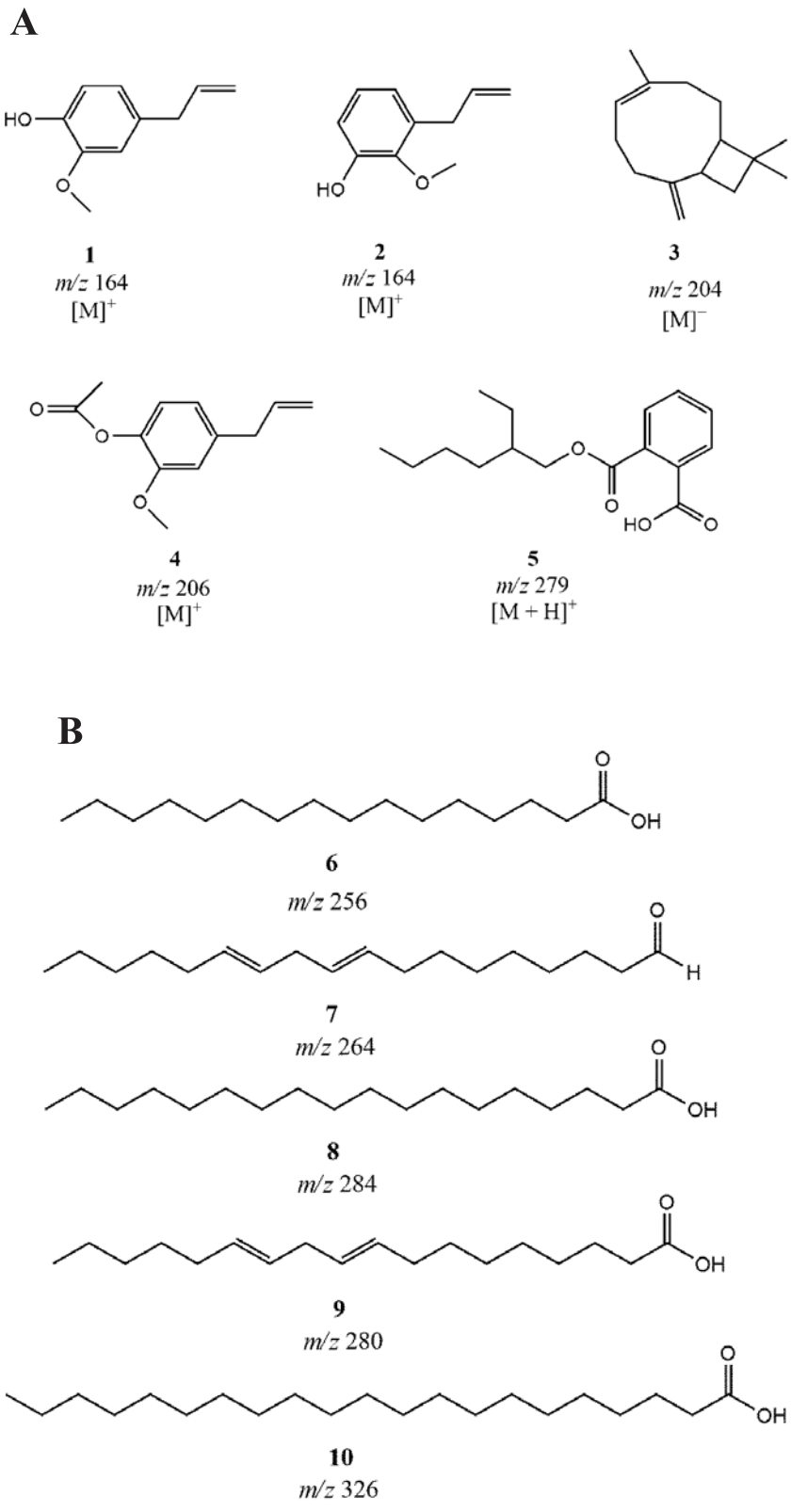


Figure 7. Structures of identified compounds from bud, leaf and stem of *E. caryophyllata* [Aromatic phenols, acids and caryophyllene (**A**), long chain aliphatic acids (**B**)]

solvent extracts (Table 2). The effect was comparable to that of ascorbic acid (0.03 ± 0.02 mg/mL) and gallic acid (0.05 ± 0.01 mg/mL).

Furthermore, our results indicated that the extracts of all the parts with the exception of the root, possessed the ability to reduce $Fe^{3+}$ to $Fe^{2+}$ (in terms of percentage gallic acid equivalent), which is comparable to that of ascorbic acid (Fig. 2). The EtOH extracts demonstrated a significantly ($p < 0.05$) higher activity compared to other solvent extracts in a dose-dependent manner. Bud (A), leaf (B) and stem (C) EtOH extracts showed higher reducing power compared to other solvent extracts. Similarly, the highest activity was exhibited by the EtOH extract of the bud, which is significantly higher compared to ascorbic acid. The extent of the reducing power ($Fe^{3+}$ to $Fe^{2+}$) by different parts of *E. caryophyllata* is in the order: bud > leaf > stem > root.

In addition, the EtOH extracts of the bud (A), leaf (B) and stem (C) exhibited significantly ($p < 0.05$) higher inhibitory effects toward hemoglobin glycosylation (Fig. 3). The $IC_{50}$ values recorded were 0.17 ± 0.020, 0.83 ± 0.04 and 1.03 ± 0.42 mg/mL for the bud, leaf and stem, respectively (Table 2). Similarly, various extracts from the root showed no inhibitory effect towards hemoglobin glycosylation. The leaf and stem EtOH extracts recorded higher $IC_{50}$ values than the bud EtOH extracts although not significantly different ($p < 0.05$) in comparison to gallic acid ($IC_{50}$ values: 0.20 ± 0.01 mg/mL).

Figure 4 shows the α-amylase inhibitory effect of various extracts from *E. caryophyllata* parts. Only the bud EtOH extract exhibited a significantly ($p < 0.05$) lower $IC_{50}$ value (0.20 ± 0.02 mg/mL) compared to acarbose ($IC_{50}$ value: 4.91 ± 0.80 mg/mL). The inhibitory effects observed on the leaf and stem EtOH extracts as well as the bud ethyl acetate and aqueous extracts did not differ significantly. The $IC_{50}$ values demonstrated by these extracts were significantly higher compared to acarbose (4.91 ± 0.80 mg/mL). In addition, ethyl acetate extracts from various parts showed lower $IC_{50}$ values compared to the aqueous extracts (Table 2). No activity was recorded with various solvent extracts from the root.

Similarly, all the solvent extracts with the exception of the root extracts demonstrated dose-dependent inhibition of α-glucosidase (Fig. 5). Significantly ($p < 0.05$) lower $IC_{50}$ values were exhibited by the EtOH extracts of the bud (0.03 ± 0.01 mg/mL), leaf (0.74 ± 0.02 mg/mL), and stem (6.89 ± 0.11 mg/mL) and by the ethyl acetate extract (0.68 ± 0.03 mg/mL) of the bud compared to other solvent extracts (Table 2). The $IC_{50}$ values demonstrated by different parts are in the order of bud > leaf > stem. The ethyl acetate extract of the stem showed the least α-glucosidase inhibitory effect, having the highest $IC_{50}$ value.

Based on the results obtained, the EtOH extracts of the bud, leaf and stem showed consistently higher activity compared to other solvent extracts and was thus subjected to GC-MS analysis to determine the phytochemicals present in this extract. From the results obtained, several peaks were observed in the chromatograms (Fig. 6). Peaks detected were compared with the data available in the NIST library and the compounds detected correspond to aromatic phenols (**1, 2**), caryophyllene (**3**), aromatics containing ether, ester and acid moieties (**4, 5**) and long chain aliphatic acids (**6**-**10**) (Table 3; Fig. 7). Eugenol (**1**) and caryophyllene (**3**) were detected in all the extracts analyzed and 2-acetyl-4-(2-propenyl)anisole (**4**) and 1,2-benzenedicarboxylic acid mono (2-ethylhexyl) ester (**5**) were detected in the EtOH extracts of the bud and leaf. The long chain aliphatic acids (**6**-**10**) were present in the EtOH extract of the bud alone.

## DISCUSSION

The present study investigated and compared, for the first time, the antioxidative and antidiabetic effects of various solvent extracts of *E. caryophyllata* parts *in vitro*. This is the first report of the potential of plant parts other than the buds (leaf, root and stem) as antidiabetic and antioxidative agents. From the results of this study, it is evident that EtOH extracts have higher yields and contain a higher total polyphenol and flavonoid content in comparison to other solvent extracts. This is consistent with previous findings that ethanol is the best solvent for the extraction of a maximum yield of polyphenols compared to other solvents (28). The amount of total polyphenols and flavonoid content was in the order of bud > leaf > stem > root (Table 1). A possible explanation could be linked to several factors including genetic and environmental factors (nature of the soil, high temperature and rainfall) in addition to growth or maturation stages (29, 30). Although no correlation analysis was carried out in this study, previous studies strongly correlate antioxidative effect to total polyphenol contents (31).

Methods adapted to assess the antioxidative effect of various parts of *E. caryophyllata* include among others, the DPPH radical scavenging assay, a widely used method for assessing the antioxidant status of compounds or plant products. In addition,

calculated $IC_{50}$ values were used to demonstrate the extent of scavenging power for different parts of the plant. The lower the $IC_{50}$ values the higher the scavenging activity. More importantly, the consistently lower $IC_{50}$ value exhibited by the EtOH extracts, comparable to standard antioxidants (ascorbic acid and gallic acid) (Fig. 2) suggest that the extracts possess compounds with high radical-quenching ability that could terminate free radical activities. This is consistent with previous studies (32-34).

The ferric reducing power which reflects electron donating capacity of various extracts has been used to assess the antioxidative status of several natural products. In this study, the EtOH extracts from the bud, leaf and stem demonstrated higher activity and therefore possessed phytochemicals that cause the reduction of $Fe^{3+}$ to $Fe^{2+}$, which is monitored by measuring the formation of Perlis prussian blue at 700 nm.

To further explore the antioxidative potential of various extracts, their ability to inhibit glycosylation of hemoglobin was determined. Glycosylation is a term used to describe the non-enzymatic reaction between reducing sugars and proteins (hemoglobin, albumin) and usually contributes enormously to the formation of advanced glycation end products (35). Consequently, it is evident from the results obtained that the EtOH extracts depicted lower $IC_{50}$ values, comparable to that of standard antioxidants used, indicating higher radical scavenging and anti-glycosylation activity (Table 2). This could be linked to the active principles present and the differences observed could be due to variation and concentration of the phytochemicals present in each part.

Moreover, it is an established fact that α-amylase and α-glucosidase inhibitors from natural sources play a significant role in diabetic management and control. This is achieved *via* a decrease in postprandial hyperglycemia through inhibition of α-amylase and α-glucosidase actions (16). However, for effective control of postprandial hyperglycemia, moderate α-amylase inhibition and potent α-glucosidase inhibition provide better options for controlling the availability of dietary glucose for absorption in the intestinal tract (36). This is due to adverse effects associated with strong α-amylase inhibition such as abdominal distension, flatulence, bowel necrosis and diarrhoea (37). In this study, various solvent extracts demonstrated mild α-amylase inhibition and potent α-glucosidase inhibition, indicating a potential role as an anti-diabetic agent. The inhibitory effects of *E. caryophyllata* bud reported by Adefegha and Oboh (16) correspond with the results of this study. Furthermore, various solvent extracts from the leaf and stem could be good substitutes for the bud as potential antidiabetic agents, as the bud is being used locally in most parts of the world.

Phytochemical analysis of the most active parts resulted in the identification of compounds with potential medicinal usage (20). For example, eugenol (**1**) has already been implicated with a wide array of therapeutic application such as antioxidative, antidiabetic and antimicrobial effects. Interestingly, eugenol (**1**) and caryophyllene (**3**) were present in all parts while others like 2-acetyl-4(2-propenyl) anisole (**4**) appear in the bud and leaf but not the stem. The availability of eugenol (**1**) in most parts of *E. caryophyllata* has already been reported (38-40). Additionally, long chain aliphatic acids (**6**-**10**), also detected in the bud could synergistically or independently contribute to the observed higher activities of this part compared to others. Furthermore, the hydroxyl group present in compounds **1** and **2** could directly or indirectly be the key feature that contributed to the higher antioxidative and antidiabetic effects depicted by the bud, leaf and stem extracts (Fig. 7). The low reduction potentials of phenolics, hydroxyls and other related compounds inactivate and terminate the initiation and propagation of chain reactions associated with oxidative damage (41). In a similar way, phenolics and hydroxyls were reported to interfere with some surface amino acid side chains in both α-amylase and α-glucosidase structures (42). This causes some conformational changes on the enzyme structure, thereby decreasing their actions and causing reduction on blood glucose levels and subsequently reduced postprandial hyperglycemia.

## CONCLUSIONS

In conclusion, various solvent extracts from the bud, leaf and stem of *E. caryophyllata* possessed antioxidative as well as antidiabetic effects *in vitro* while the root extracts showed very low or no significant effects in the same assays. The effects of the leaf extracts were comparable to that of the bud and could therefore serve as a good substitute for various culinary and medicinal potentials of the bud. Hence, it is recommended that bioassay-guided fractionation of the EtOH extracts could be done in order to fully investigate the *in vivo* antidiabetic and antioxidative effects of this extract.

### Declaration of interest

There is no conflict of interest within this article.

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# PRELIMINARY STUDY ON THE COMPOSITION OF VOLATILE FRACTION OF FRESH FLOWERS AND LEAVES OF *ROBINIA PSEUDOACACIA* L. GROWING IN POLAND

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**Abstract:** The essential oils obtained by hydrodistillation from fresh flowers and leaves of *Robinia pseudoacacia* L. were analyzed by GC-FID-MS in respect to their chemical composition. Eighty volatile compounds (96.3% of the oil) contained in the flowers and sixty-three compounds (97.5%) derived from the leaves were identified. The major components in the flower oil were linalool (20.4%), 3-methyltetradecane (16.5%), hexahydrofarnesyl acetone (14.9%) and 1-octen-3-ol (13.4%). The leaf oil comprised an abundance of 1-octen-3-ol (57.9%), phytol (9.1%), (*Z*)-3-hexenyl acetate (6.0%) and (*Z*)-3-hexen-1-ol (5.2%).

**Keywords:** *Robinia pseudoacacia*, Fabaceae, essential oil composition, linalool, 1-octen-3-ol

The genus *Robinia* L. (Fabaceae) commonly known as locust comprises 10 species of trees and shrubs characterized by white or pink flowers with intensive, distinctive, sweet aroma (1, 2). *Robinia pseudoacacia* L. (black locust), originally native to the south-eastern USA, is widely distributed as wild and cultivated species growing in temperate regions throughout the world (1-3). As a species with little requirements, it has also adapted successfully to a diverse range of habitats in Poland (4). It is extensively used as a shade tree or for ornamental purposes. Black locust as large-sized tree, growing to around 20 meters tall can be distinguished by numerous white flowers, grouped in pendulous racemes, and pinnate leaves with nine to nineteen leaflets and a pair of short thorns at the base (1-3). Mainly the flowers of black locust are used in traditional medicine as diuretic, spasmolytic, sedative and cholagogic agents and relieve inflammation of the kidneys and biliary ducts (5). This biological activity can be connected with the presence of a non-volatile fraction which includes flavonoids such as robinin, biorobin, rhamnorobin, acaciin and other triglycosides of apigenin, luteolin and diosmetin with glucose and rhamnose (6-10). Moreover, the presence of coumarins (11), sterols (12) and fatty acids (13) has been reported.

The available literature indicates that black locust flowers, attracting a special interest due to its gentle fragrance, contain the essential oil whose composition depends on geographical region. The major volatile constituents of the flowers varied greatly among samples collected in China. According to Xie et al. linalool, *(Z)*-β-ocimene, *(E)*-β-bergamotene and formanilide are the main constituents isolated from the flowers by SPME method (14). In the essential oil obtained by Long et al. phenylethyl alcohol, linalool and geraniol were recognized as predominant compounds (15). Considering the results revealed by Wang et al. the main ones were limonene and γ-terpinene (16). The oil from the flowers grown in Iran was dominated by ethyl hexanoate, heptadecane and virdiflorol (17), whereas in the oil from the flowers collected in the USA, the main constituents were δ-3-carene, linalool and anthranilic aldehyde (18). This wide range of constituents found in the literature prompted the present study, whose aim is to determine the volatile constituents released from fresh flowers and leaves of *Robinia pseudoacacia* L. collected in Poland.

## EXPERIMENTAL

### Plant material

Fresh flowers and leaves of *Robinia pseudoacacia* L. were collected by hand in May 2012 from a ten-year-old black locust tree naturally growing in central Poland (Łódź). The voucher specimens

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(RPA-F12 and RPA-L12), identified and authenticated by Professor M. Wolbiś, were deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Łódź.

**Essential oil isolation**

Fresh flowers (800 g) and leaves (700 g) were hydrodistilled separately for 3 h using a Clevenger type apparatus with *n*-pentane (1 mL) as collecting solvent. The essential oils were dried over anhydrous sodium sulfate and stored at 4°C until analyzed.

**Analysis of essential oil**

GC-FID-MS analyses were performed using a Trace GC Ultra apparatus (Thermo Electron Corporation) equipped with FID and MS DSQ II detectors and FID-MS splitter (SGE). Apolar capillary column Rtx-1MS (Restek), 60 m × 0.25 mm i.d., film thickness 0.25 μm; temperature program, 50-300°C at 4°C/min, SSL injection temperature 280°C, FID temperature 300°C, split ratio 1 : 20, carrier gas helium at a regular pressure 300 kPa. Polar capillary column TGWax-Gold (Thermo Scientific), 30 m × 0.25 mm i.d., film thickness 0.25 mm, temperature program 50-240°C (30 min) at 4°C/min; SSL injection temp. 250°C FID temperature 260°C; carrier gas, helium; 0.8 mL/min; split ratio 1 : 20. Mass spectra were acquired over the mass range 30-400 Da, ionization voltage 70 eV, ion source temperature 200°C.

Individual components were identified by the comparison of their MS spectra and GC retention indices (relative to *n*-alkane $C_8$-$C_{26}$) with those stored in NIST 98.1, Wiley Registry of Mass Spectral Data, 8th edn. and Mass Finder 3.1 libraries. The relative amount of the individual components was calculated from peak area without applying an FID response factor correction.

## RESULTS AND DISCUSSION

The hydrodistillation of fresh flowers and leaves of *Robinia pseudoacacia* L. gave only trace yield of the oils, thus the volatiles were trapped in *n*-pentane. The previous report indicated that the flowers of *R. pseudoacacia* are poor in essential oil whose content is less than 0.001 mL/kg (19). In the present study, ninety-nine essential oil compounds were identified according to their retention indices (RI) and mass spectra. The list of identified volatile constituents, their retention indices (RI) and the percentages, in order of their elution from Rtx-1MS column are given in Table 1.

Chemical analysis of the flower essential oil revealed the presence of eighty components, representing 96.3% of the total oil. In the flowers, the monoterpene fraction amounted to 30.1% of the total composition and was particularly characterized by linalool (20.4%) with the following predominant compounds: geraniol (2.0%), terpinen-4-ol (1.7%) and α-terpineol (1.5%). The sesquiterpene fraction (13.1%) consisted of sesquiterpene alcohols and hydrocarbons with (*E,E*)-farnesol (4.6%), (*E*)-nerolidol (2.3%), (*E,E*)-α-farnesene (2.0%), β-bisabolol (1.5%) and (*E,Z*)-α-farnesene (1.1%) being the main compounds. Considerable was the content of hydrocarbons (20.2%) and carbonyl compounds (16.0%), particularly represented by 3-methyltetradecane (16.5%) and the C-18 ketone hexahydrofarnesyl acetone (14.9%). Other components of the essential oil were aliphatic alcohols (14.2%) including 1-octen-3-ol (13.4%) as the predominant compound.

The gentle fragrance characteristic of *R. pseudoacacia* flowers is mainly due to the presence of large amounts of linalool, 1-octen-3-ol and hexahydrofarnesyl acetone in the essential oil. Linalool depending on isomeric forms (3*R*-(-)- or 3*S*-(+)-linalool) has a lavender, lily-of-the-valley or more herbaceous and musty green odor, respectively. Furthermore, the enantiomeric distribution of linalool showed that 3*R*-(-) form is much more common in essential oils than its second isomer (20). Hexahydrofarnesyl acetone is associated with long lasting fresh jasmine fragrance (21, 22). In contrast, 1-octen-3-ol that has been cited as a marker for rotten grapes and wines, constitutes one of the main characteristic fungal odor (23, 24).

In the case of the leaf essential oil, sixty-three components were identified, which represented 97.5% of the total detected constituents. In the leaves, aliphatic alcohols $C_6 - C_{16}$ (65.1%) prevailed, particularly unsaturated chain compounds such as 1-octen-3-ol (57.9%) and (*Z*)-3-hexen-1-ol (5.2%). Besides, the leaves were characterized by the presence of (*E*)- and (*Z*)-3-hexen-1-ols and their ester derivatives (6.5%), which were not detected in the flowers. Within this group, (*Z*)-3-hexenyl acetate (6.0%), (*Z*)-3-hexenyl butyrate (0.1%), (*Z*)-3-hexenyl lactate (0.1%), (*Z*)- and (*E*)-3-hexenyl caproates (0.1%), (*Z*)-3-hexenyl benzoate (< 0.05%) and (*Z*)-3-hexenyl salicylate (< 0.05%) were identified. The other main components of leaf essential oil were terpenes (17.4%), among which monoterpene and sesquiterpene fractions amounted to 7.7% and 0.6%, respectively. The monoterpenes were characterized by a high content of linalool (3.5%) and geraniol (3.3%). Also considerable were phytol (9.1%) and hexahydrofarnesyl acetone contents (1.2%).

Table 1. Retention indices (RI) and the percentage composition of identified compounds in the essential oils from the flowers and leaves of *Robinia pseudoacacia* L.

| **Compound**[a] | **RI**$_{Rtx}$[b] | **RI**$_{lit}$[c] | **RI**$_{Wax}$[b] | **Flowers** | **Leaves** |
|---|---|---|---|---|---|
| *E*)-3-hexen-1-ol | 837 | 840 | 1363 | – | 0.1 |
| (*Z*)-3-hexen-1-ol | 840 | 846 | 1384 | – | **5.2** |
| Hexanol | 854 | 860 | 1351 | 0.1 | 0.6 |
| (*E*)-3,5,5,-trimethyl-2-hexene | 960 | 968 | 1484 | – | 0.9 |
| 1-Octen-3-ol | 968 | 962 | 1452 | **13.4** | **57.9** |
| 2,3-Dehydro-1,8-cineole | 979 | 985 | 1192 | 0.4 | – |
| 3-Octanol | 984 | 981 | 1391 | 0.1 | 0.4 |
| (*Z*)-3-hexenyl acetate | 991 | 987 | 1320 | – | **6.0** |
| Decane | 1000 | 1000 | 1000 | tr | 0.1 |
| α-Terpinene | 1009 | 1013 | 1183 | 0.1 | – |
| Limonene | 1022 | 1025 | 1220 | tr | tr |
| (*Z*)-β-ocimene | 1029 | 1029 | 1237 | tr | – |
| (*E*)-β-ocimene | 1039 | 1041 | 1254 | 0.9 | – |
| γ-Terpinene | 1049 | 1051 | 1248 | 0.2 | – |
| *trans*-Sabinene hydrate | 1054 | 1053 | 1465 | 0.1 | – |
| (*E*)-2-octen-1-ol | 1054 | 1059 | 1614 | 0.2 | 0.5 |
| Octanol | 1058 | 1063 | 1555 | – | 0.6 |
| *trans*-Linalool oxide (furanoid) | 1058 | 1058 | | 0.6 | – |
| *cis*-Linalool oxide (furanoid) | 1073 | 1072 | 1473 | 0.1 | tr |
| Terpinolene | 1079 | 1082 | 1287 | 0.1 | – |
| Nonanal | 1084 | 1084 | 1398 | 0.3 | 0.7 |
| Linalool | 1090 | 1086 | 1550 | **20.4** | 3.5 |
| Undecane | 1100 | 1100 | 1100 | tr | tr |
| *trans*-p-Mentha-2,8-dien-1-ol | 1105 | 1113 | 1628 | 0.2 | – |
| *cis*-p-Mentha-2-en-1-ol | 1107 | 1108 | 1565 | 0.2 | – |
| *cis*-p-Mentha-2,8-dien-1-ol | 1118 | 1125 | 1670 | 0.1 | – |
| *trans*-p-Mentha-2-en-1-ol | 1124 | 1123 | 1622 | 0.1 | – |
| *cis*-Verbenol | 1126 | 1132 | 1657 | 0.1 | – |
| *trans*-Verbenol | 1129 | 1136 | 1678 | 0.5 | – |
| p-Mentha-1,5-dien-8-ol | 1148 | 1148 | 1727 | 0.2 | – |
| 1,4-Dimethyl-δ-3-tetrahydroacetophenone | 1155 | 1152 | 1565 | 0.1 | 0.1 |
| Nonanol | 1159 | 1156 | 1657 | 0.1 | 0.3 |
| Terpinen-4-ol | 1163 | 1164 | 1604 | 1.7 | 0.1 |
| (*Z*)-3-hexenyl butyrate | 1170 | 1170 | 1465 | – | 0.1 |
| α-Terpineol | 1174 | 1176 | 1698 | 1.5 | 0.4 |
| Safranal | 1176 | 1182 | 1652 | – | 0.1 |
| Decanal | 1186 | 1180 | 1503 | 0.5 | 0.5 |
| (*E,E*)-2,6-dimethyl-3,5,7-octatriene-2-ol | 1189 | 1187 | 1821 | 0.2 | – |
| Nerol | 1212 | 1210 | 1799 | 0.4 | 0.1 |
| (*Z*)-3-hexenyl lactate | 1217 | 1187 | 1722 | – | 0.1 |
| Geraniol | 1238 | 1235 | 1845 | 2.0 | 3.3 |
| Decanol | 1262 | 1259 | 1759 | – | tr |
| Dihydroedulan II | 1284 | 1290 | 1524 | 0.2 | 0.1 |

Table 1. Cont.

| Compound[a] | $RI_{Rtx}$[b] | $RI_{lit}$[c] | $RI_{Wax}$[b] | Flowers | Leaves |
|---|---|---|---|---|---|
| Theaspirane (isomer 1) | 1291 | 1299 | 1503 | 0.1 | 0.4 |
| Tridecane | 1299 | 1300 | 1299 | tr | tr |
| Theaspirane (isomer 2) | 1305 | 1313 | 1545 | 0.1 | 0.4 |
| Methyl anthranilate | 1320 | 1328 | 2248 | 0.3 | – |
| (*E*)-β-damascenone | 1363 | 1363 | | tr | – |
| (*Z*)-3-hexenyl caproate | 1364 | 1362 | 1655 | – | 0.1 |
| (*E*)-3-hexenyl caproate | 1366 | 1368 | 1660 | – | 0.1 |
| (*Z*)-jasmone | 1371 | 1371 | 1950 | 0.1 | 0.3 |
| Hexahydropseudoionone | 1388 | 1389 | 1689 | 0.1 | – |
| Tetradecane | 1399 | 1400 | 1406 | – | tr |
| Geranyl acetone | 1432 | 1434 | 1856 | 0.1 | – |
| Sesquisabinene A | 1436 | 1435 | 1644 | 0.1 | – |
| (*E*)-β-farnesene | 1447 | 1446 | 1665 | 0.1 | – |
| 3-Methyltetradecane | 1468 | 1462 | 1443 | **16.5** | 0.4 |
| β-Ionone | 1468 | 1468 | 1944 | – | 0.1 |
| γ-Curcumene | 1473 | 1475 | 1690 | 0.2 | – |
| (*E*,*Z*)- α-farnesene | 1479 | 1475 | 1685 | 1.1 | tr |
| *trans*-β-Bergamotene | 1483 | 1480 | 1580 | 0.1 | – |
| (*E*,*E*)-α-farnesene | 1497 | 1498 | 1749 | 2.0 | 0.1 |
| Pentadecane | 1499 | 1500 | 1500 | – | tr |
| β-Curcumene | 1502 | 1503 | 1741 | 0.4 | – |
| (*Z*)-γ-bisabolene | 1508 | 1505 | 1730 | 0.1 | – |
| (*E*)-α-bisabolene | 1533 | 1530 | 1773 | 0.1 | – |
| (*E*)-nerolidol | 1549 | 1553 | 2039 | 2.3 | 0.3 |
| (*Z*)-3-hexenyl benzoate | 1554 | 1554 | 2131 | – | tr |
| *cis*-Sesquisabinene hydrate | 1566 | 1565 | 2084 | tr | – |
| Hexadecane | 1598 | 1600 | 1600 | – | tr |
| *trans*-Sesquisabinene hydrate | 1600 | 1598 | 2112 | 0.1 | – |
| α-Acorenol | 1620 | 1623 | 2170 | 0.2 | – |
| (*Z*)-3-hexenyl salicylate | 1651 | 1648 | 2272 | – | tr |
| β-Bisabolol | 1659 | 1659 | 2155 | 1.5 | – |
| α-Bisabolol | 1668 | 1673 | | 0.1 | – |
| (*Z*,*E*)-farnesol | 1682 | 1687 | 2311 | tr | – |
| Heptadecane | 1698 | 1700 | 1700 | – | tr |
| (*E*,*E*)-farnesol | 1704 | 1710 | 2354 | 4.6 | 0.3 |
| (*E*,*Z*)-farnesol | 1708 | 1718 | | 0.1 | – |
| Octadecane | 1798 | 1800 | 1798 | 0.1 | 0.1 |
| Hexahydrofarnesyl acetone | 1835 | 1823 | 2135 | **14.9** | 1.2 |
| 6,10,14-Trimethylpentadecan-2-ol | 1841 | 1843 | 2096 | 0.2 | – |
| Hexadecanol | 1867 | 1866 | 2374 | 0.2 | – |
| Nonadecane | 1898 | 1900 | 1900 | tr | tr |
| Methyl palmitate | 1909 | 1909 | 2216 | 0.4 | 0.1 |
| Hexadecanoic acid | 1946 | 1942 | 2618 | 0.2 | 0.4 |
| Methyl linolate | 2073 | 2087 | | 0.3 | tr |

Table 1. Cont.

| Compound[a] | $RI_{Rtx}$[b] | $RI_{lit}$[c] | $RI_{Wax}$[b] | Flowers | Leaves |
|---|---|---|---|---|---|
| Methyl linolenate | 2077 | 2092 | 2566 | 0.3 | tr |
| Heneicosane | 2099 | 2100 | 2096 | 0.1 | 0.1 |
| Phytol | 2102 | 2114 | 2601 | 0.5 | **9.1** |
| Methyl stearate | 2110 | 2113 | 2422 | 0.1 | 0.1 |
| Stearyl acetate | 2193 | 2208 | 2506 | 0.2 | 0.1 |
| Docosane | 2199 | 2200 | 2200 | – | tr |
| Tricosane | 2298 | 2300 | 2293 | 0.3 | 0.7 |
| Eicosanyl acetate | 2393 | 2406 | 2585 | 0.1 | tr |
| Tetracosane | 2399 | 2400 | 2392 | tr | tr |
| Docosanal | 2412 | 2426 | 2618 | 0.1 | 0.1 |
| Pentacosane | 2501 | 2500 | 2499 | 1.1 | 0.6 |
| Hexacosane | 2610 | 2600 | 2606 | 2.1 | 0.6 |
| **Total** | | | | **96.3** | **97.5** |
| Terpenes: | | | | **43.7** | 17.4 |
| Monoterpenes | | | | 30.1 | 7.7 |
| Sesquiterpenes | | | | 13.1 | 0.6 |
| Diterpenes | | | | 0.5 | 9.1 |
| Aliphatic alcohols | | | | 14.2 | **65.1** |
| (*E*), (*Z*)-3-hexen-1-ol derivatives | | | | – | 6.4 |
| Aliphatic hydrocarbons | | | | 20.2 | 3.7 |
| Carbonyl compound | | | | 16.0 | 2.9 |
| Others | | | | 2.2 | 2.0 |

[a] Compounds are listed in order of their elution from Rtx-1MS column [b] $RI_{Rtx}$, $RI_{Wax}$ – retention indices determined on Rtx-1MS and TGWax-Gold columns, respectively. [c] Literature values. – Not detected, tr – trace (percentage value less than 0.05%)

The literature review showed that linalool, commonly found in fragrances and essential oils, accounted for 20.4% (the flowers) and 3.5% (the leaves) of the present oils has been the subject of numerous studies investigating its anti-inflammatory, spasmolytic, sedative, anesthetic and antimicrobial activity. Moreover, linalool could enhance the permeability of a number of drugs through biological tissues like skin or mucus membranes (25, 26). Hexahydrofarnesyl acetone, present in flower oil as one of the major compound (14.9%), could be responsible for its antifungal, antibacterial and cytotoxic activity (27, 28). 1-Octen-3-ol prevailing in the present leaf and flower oils (57.9, 13.4%, respectively) was also considered as bioactive compound and indicated acaricidal and antifungal activity (29, 30). Moreover, green leaf volatiles including (*Z*)-3-hexen-1-ols and their ester derivatives characteristic for leaf oil may act as host-plant attractants, fruit ripeness indicators or as defensive secretions (31, 32).

Comparing previously available data, this report confirms that the composition of the essential oil of *R. pseudoacacia* flowers collected in Poland is not similar to that obtained from various regions of China, Iran and the USA (14-18). Although certain constituents of the oils such as (*Z*)-β-ocimene, limonene and γ-terpinene, detected by other researches as predominant, were also identified in the Polish essential oil, their concentrations were less than 0.2%. Exclusively, linalool and geraniol, at concentrations of 3.1–33.1% and 1.0– 2.1%, respectively, were among the major components also detected in flower oils from Poland, China and the USA. The chemical variations of the essential oils of *R. pseudoacacia* flowers collected from different locations might have been due to environmental factors (geographical, climatic and seasonal), storage duration of plant material and type of the methods used to produce the essential oils. Due to the fact, that in the current study the volatile constituents were investigated for plant samples collected in one

year and from a single plant area, the present results could be considered as preliminary and will require further comparative analyses for the flowers and leaves derived at different stages of plant development and from various geographical regions of Poland. Therefore, the studying variability of natural population of *R. pseudoacacia* in various countries may have a great role in reliable evaluation of the essential oil composition of this species.

## CONCLUSIONS

Summing up, the essential oil of *R. pseudoacacia* flowers collected in Poland was found to be rich in linalool (20.4%), 3-methyltetradecane (16.5%), hexahydrofarnesyl acetone (14.9%) and 1-octen-3-ol (13.4%). The leaf oil, which was analyzed in *R. pseudoacacia* for the first time, contained greater amounts of 1-octen-3-ol (57.9%), phytol (9.1%), (*Z*)-3-hexenyl acetate (6.0%) and (*Z*)-3-hexen-1-ol (5.2%). The qualitative profiles of the essential oils extracted from the flower and leaf are largely similar even though the leaves additionally include aliphatic alcohols $C_6$ – $C_{16}$ and their ester derivatives. The further comprehensive studies of essential oil of *R. pseudoacacia* flowers and leaves of Polish origin would be desirable to clarify the relationship between variations of the essential oil composition and various geographical factors as well as morphological features in natural Polish populations.

**Acknowledgment**

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# EFFECT OF TCM *YINHUANGSAN* ON RAT'S DIABETIC ULCER HEALING MORPHOLOGY AND RECOVERY FACTORS

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**Abstract:** The research was done to study the role of the application of Traditional Chinese Medicines (TCM) external treatment, "*Yinhuangsan*", in the process of the wound tissue healing when it is used to intervene in the diabetic foot ulcers, and then to explore the mechanism of the rat diabetic ulcer healing. For the establishment of diabetic ulcer model, rats were randomly divided into control group, western medicine group, and TCM group, each consisted of 10 rats, respectively, corresponding to no drug, metronidazole and *Yinhuangsan* treatment. After 10 days of modeling, analyses were made to detect the contents of serum AGEs, inflammatory factor and growth factor, using proliferating cell nuclear antigen (PCNA) staining to detect the number of fibroblast in the wound granulation tissue, using CD34 staining to detect the number of new capillaries in granulation tissue and hematoxylin and eosin (HE) staining was used to observe morphological changes. Compared with the model group, in serum of rats, advanced glycosylation end products (AGEs) and tissue necrosis factor (TNF-$\alpha$), interleukin-1 (IL-1), and C-reactive protein (CRP) were reduced after drug intervention, newborn capillaries, basic fibroblast growth factor (bFGF), endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF) were improved after drug intervention, newborn capillaries, fibroblasts were increased ($p < 0.05$) in granulation tissue, and TCM treatment group was more pronounced ($p < 0.05$) than western medicine treatment group. *Yinhuangsan* can significantly promote the ulcer healing in diabetes mellitus rats, because it can reduce inflammatory factor and improve growth factor in rat serum. Besides, *Yinhuangsan* can reduce AGEs in rat serum and promote new capillaries and fibroblasts proliferation in the granulation tissue.

**Keywords:** *Yinhuangsan*, diabetic ulcer healing, inflammatory factor, growth factor, AGEs

Diabetic foot is a complicated disease with a few theories of mechanism. In recent years, the lipid and glucose toxicity has become the research interest, especially the accumulation of AGEs (advanced glycosylation end products) on skin tissue. It has resulted in the draw of intense attention of researchers to histocytology (1). Research shows that high blood sugar and the accumulation of AGEs not only impair the capillaries and peripheral vessel, but also reduce the expression of multiple blood vessels and neurotrophic factors, which could be the important reason for the difficulty in diabetic foot ulcer. The healing process of diabetic foot ulcer is dynamic involving three continuous and overlapping stages during reparative process: inflammatory phase, granulation tissue forming phase and cicatrix remodeling phase (2).

In Traditional Chinese Medicines (TCM), the diabetic foot ulcer belongs to the field of gangrene and consumptive thirst. Most TCM practitioners believe that the pathophysiological mechanisms of this disease involve depletion of Qi and Yin, blood stasis blockage of the collaterals and exogenous damp and hot (3). In our opinion, the main causes of diabetic foot ulcer are damp, hot and toxicity stasis, and the TCM external treatment emphasis on clearing heat, detoxification and eliminating dampness.

*Yinhuangsan* is made up of golden cypress, lumbricus, dragon's blood and other traditional Chinese medicines (4, 5). Following the way of clearing heat, detoxification and eliminating dampness, together with importance of removing necrotic tissue and promoting granulation princi-

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ple, this medicine could be considered to promote wound healing and reduce the rate of amputation (6).

The aim of this study involves the assessment of cytobiological activity changes of AGEs, inflammatory factors (TNF-α, IL-1, hs-CRP) and growth factors (bFGF, EGF, VEGF, PDGF) in the wound healing process after applying *Yinhuangsan* to explore the related mechanisms of diabetic foot ulcer healing.

## EXPERIMENTAL

### Reagents and drugs

Streptozotocin (SZT, lot number: 20120728, CAS number: 18883-66-4) was purchased from Sigma-Aldrich, USA. Citric acid and sodium citrate were acquired from Beijing Chemical Reagent Factory (lot number 051024). Elisa kit (Shanghai Lianshuo Biotechnology Ltd.), IHC kit (Beijing Zhongshanjinqiao Biological Product Ltd.), PCNA detection kit (ZM-0213, lot number: 13132A10), and CD34 detection kit (ZM-0046, lot number: 12201110) were purchased through commercial sources.

### Experimental animal and fodder

SPF grade, SD male rats having 80-100 g weight were purchased from CAMS, China and were kept in iron cages (5 animals per cage) in controlled temperature conditions, i.e., approximately 37°C. Fodder (high fat and sugar) formula consisted of basal feed 67.5%, sucrose 20%, lard 10%, and yolk 2.5%. Water was provided *ad libitum*.

### STZ solution preparation

Solutions A and B comprising of citric acid and sodium citrate, respectively, were made. Both solutions were mixed with each other to make buffer solution with A : B = 1 : 1.32 proportion. The STZ was precisely weighed and dissolved to yield 1% concentration in the buffer solution. Attention: STZ should be compounded in dark place and in ice-bath, and it is required to be consumed in 15 min. The formula of STZ injected dose: weight × STZ presupposed dosage of administration/1000 = STZ dosage (mg) (7).

### Drug preparation

*Yinghuangsan* was produced by Beijing Kangrentang pharmacy Ltd., with golden cypress, lumbricus, dragon's blood and other medicine smashed to fine powder (went beyond No. 6 griddle). The contrast drug was metronidazole glucose injection (Beijing Shuanghe pharmacy Ltd., lot number: listed product 1102161), used for comparative analysis of data obtained from Chinese herbal drug.

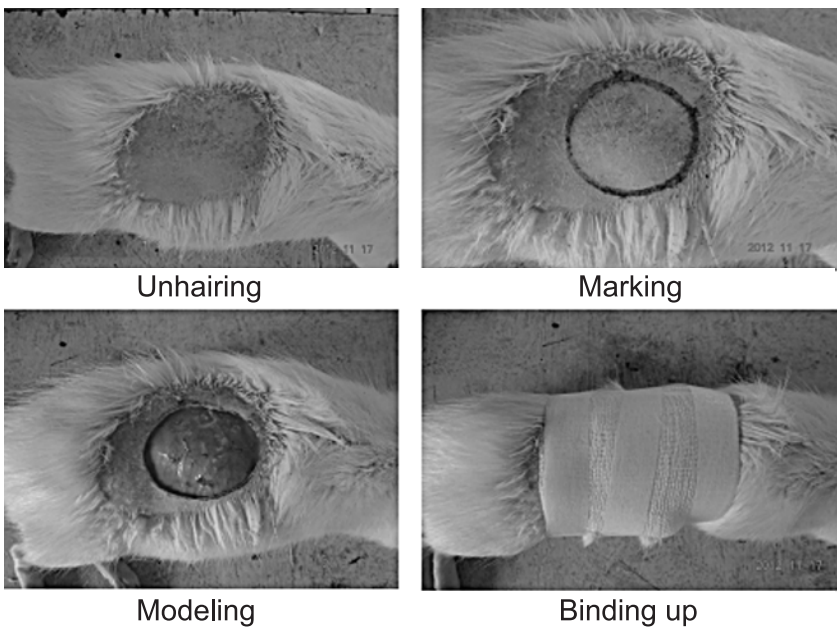


Figure 1. Diabetic ulcer rat model preparation

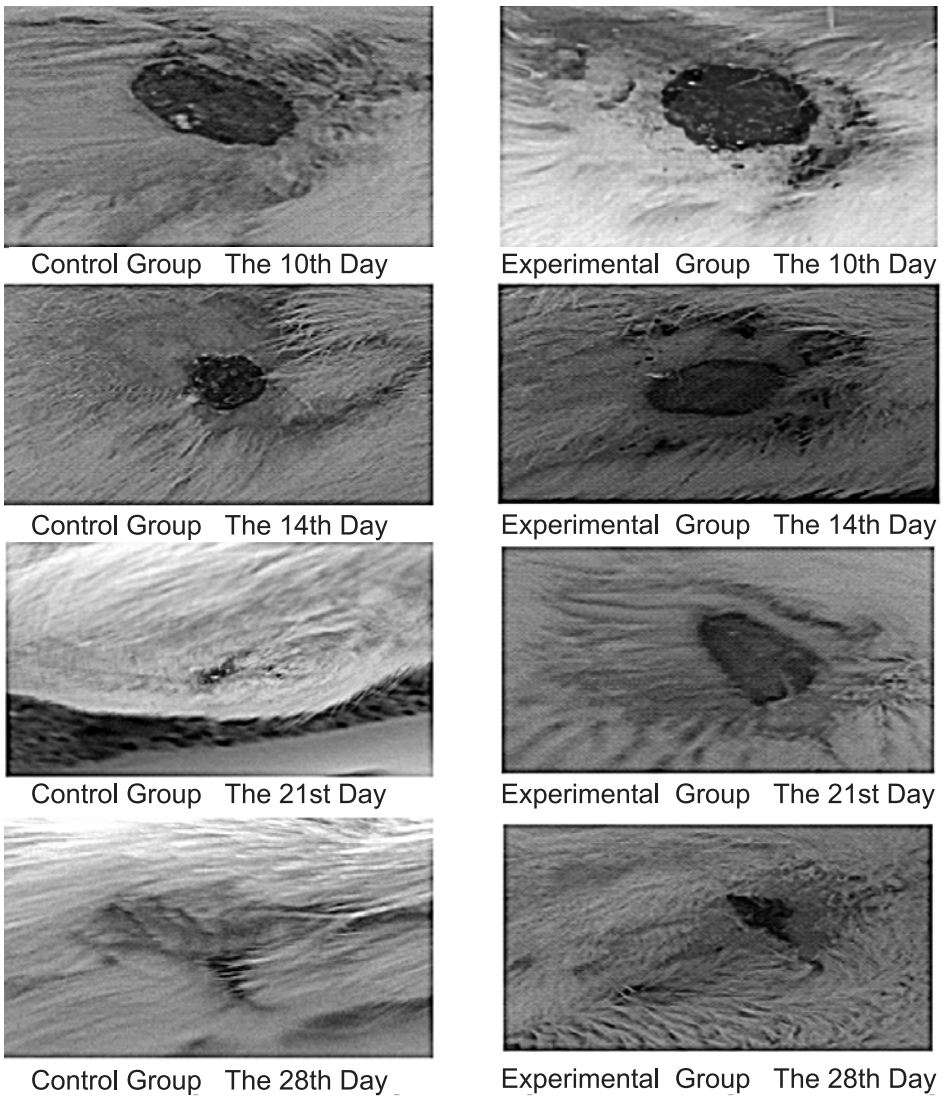


Figure 2. The wound condition of control and experimental groups

## Method

### *Model preparation of diabetic ulcer tat*

Sixty rats were chosen and fed with high fat and sugar fodder for a month. Then, STZ was injected in a quantity of 40 mg/kg body weight to their abdominal cavity to prepare diabetic rat model. To 30 model rats and 10 ordinary rats, 3.5% chloral hydrate was injected in a quantity of 1 mL/100 g to their abdominal cavity to induce narcosis. After removing long hair on their back with an electric razor, the molding area was marked with gentian violet and cut out the skin in the molding area deep into the fascia under the aseptic condition (8). The wound area needs to be covered by 6 layers of medical gauze and be banded up and fixed with medical paper tape (Fig. 1).

### *Method of administration*

Rats with diabetic ulcer were randomly divided into 3 groups: 10 rats in model control group, 10 in western medicine group, and 10 in Chinese medicine group. In addition, 10 normal SD rats were kept in blank group. Rats with wounds in the model control group and blank group were not treated since they act as positive and negative control group,

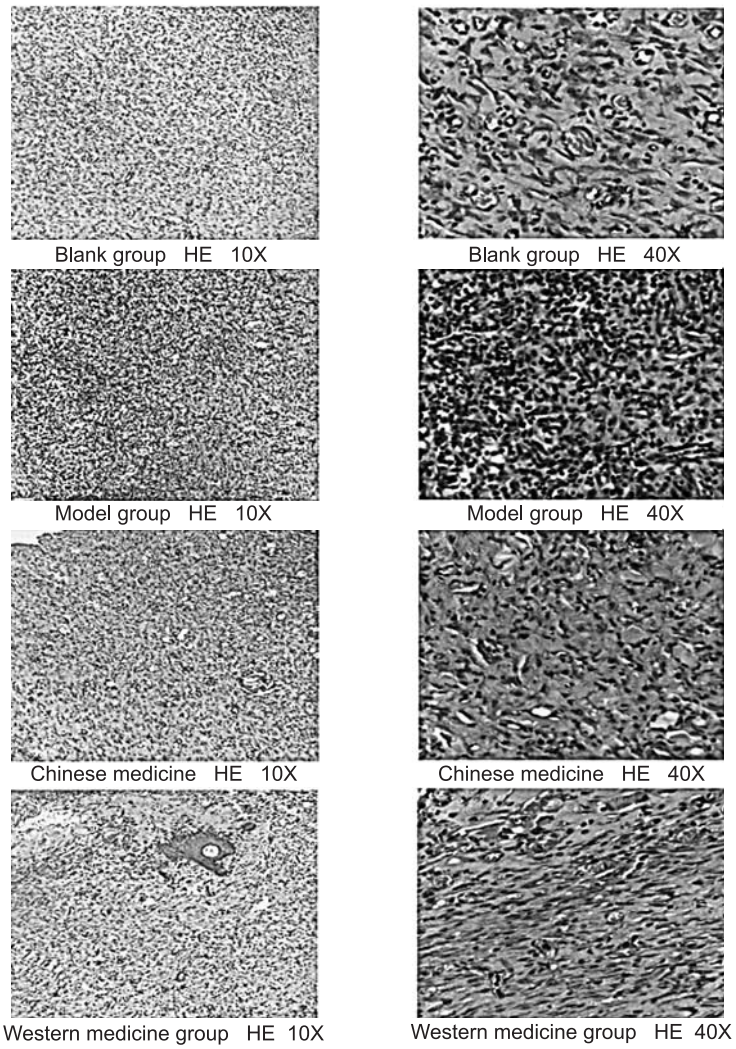


Figure 3. HE staining of paraffin sections of rat's granulation tissue

Table 1. Test of inflammation and growth factors.

| Items | Blank group | Model control group | Western medicine group | Chinese medicine group |
|---|---|---|---|---|
| n→ | 10 | 10 | 10 | 10 |
| TNF-α (ng/L) | 17.51 ± 0.39 | 21.71 ± 1.17* | 18.06 ± 0.59$^{\#}$ | 17.66 ± 0.49$^{\#}$ |
| hs-CRP (ng/L) | 0.33 ± 0.03 | 0.56 ± 0.08* | 0.42 ± 0.05$^{\#}$ | 0.35 ± 0.08$^{\#\$}$ |
| IL-1 (ng/L) | 4.99 ± 0.23 | 5.68 ± 0.43* | 4.56 ± 0.40$^{\#}$ | 4.23 ± 0.40$^{\#\$}$ |
| bFGF (ng/mL) | 3.04 ± 0.35 | 2.20 ± 0.33* | 3.5 ± 0.37$^{\#}$ | 4.47 ± 0.32$^{\#\$}$ |
| EGF (ng/mL) | 903.52 ± 25.60 | 661.34 ± 30.50* | 862.35 ± 33.94$^{\#}$ | 968.15 ± 30.02$^{\#\$}$ |
| VEGF (pg/mL) | 84.38 ± 6.26 | 65.36 ± 2.80* | 84.76 ± 2.86$^{\#}$ | 95.74 ± 3.02$^{\#\$}$ |
| PDGF (ng/mL) | 1.43 ± 0.10 | 1.25 ± 0.02* | 1.47 ± 0.09$^{\#}$ | 1.64 ± 0.04$^{\#\$}$ |
| AGEs (ng/mL) | 55.09 ± 5.82 | 72.16 ± 5.97* | 54.84 ± 6.01$^{\#}$ | 46.96 ± 2.16$^{\#\$}$ |

* Compared with blank group, $p < 0.01$, $^{\#}$ compared with model control group, $p < 0.01$ and $^{\$}$ compared with western medicine group, $p < 0.01$.

respectively. Rats in western medicine groups were applied externally gauze (4 × 4 cm) with metronidazole and glucose injection and bound up with non-woven medical tap once a day. Rats in Chinese medicine group were applied externally "Yinghuang Powder", then covered with 6 layers of gauze (4 × 4 cm) and bound up with non-woven medical tap once a day (9). All those last 10 days to observe (Fig. 2).

***Collection and test of serum sample***

Blood from rats' abdominal aorta was collected and centrifuged for 10 min at the speed of 3000 rpm. Serum separated and extracted is moved into EP tube and preserved in fridge at -20°C. The contents of TNF-α, IL-1, hs-CRP, EGF, bFGF, VEGF, PDGF, and AGEs were tested by ELISA (enzyme-linked immunosorbent assay) (10).

***Collection of granulation tissue and preparation of paraffin sections***

Granulation tissue (about 5 g) was took from the center of wound, instilled neutral PA (paraformaldehyde) and shaped, washed in clear water, soaked, dehydrated, cleared, immersed in wax and sliced into 5 μm sections (11).

***HE staining***

Paraffin sections were dewaxed by xylene, cleaned by deionized water, stained by hematoxylin, color separated by hydrochloric acid and alcohol, cleaned in water, stained by hematoxylin and eosin, dehydrated by alcohol, cleared by xylene and sealed with gum. Then, observation and comparing around the units of skin structure and functions (units of revived structure) started and multi-functional fluo-

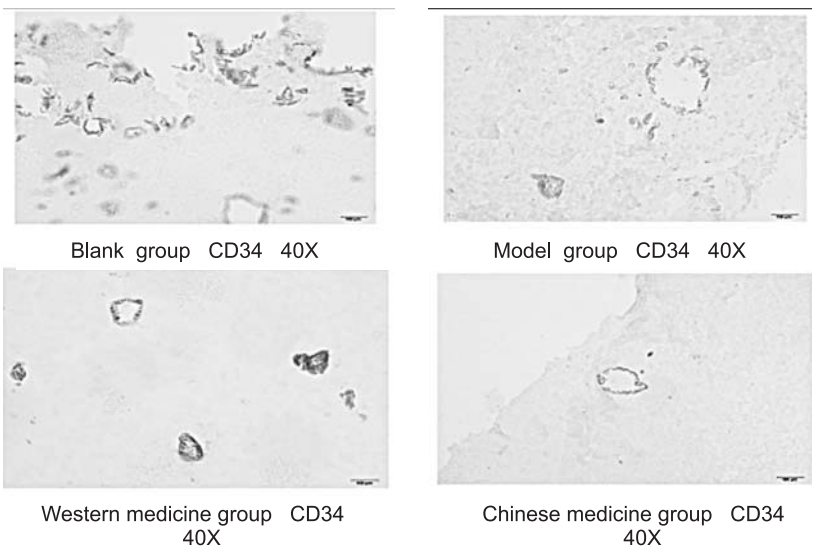


Figure 4. Immunohistochemical staining results of new blood capillaries of rat's granulation tissue

Table 2. Test of capillary density and fibroblast number.

| Items | Blank group | Model control group | Western medicine group | Chinese medicine group |
|---|---|---|---|---|
| n→ | 10 | 10 | 10 | 10 |
| New capillary density /$10^{-5}$/ $\mu m^2$ | 8.02 ± 0.48 | 4.47 ± 0.32* | 6.56 ± 0.27# | 7.13 ± 0.17$ |
| Fibroblast number per visual field | 348.24 ± 6.12 | 157.19 ± 5.23* | 259.46 ± 6.7# | 302.25 ± 4.15$ |

* Compared with blank group, $p < 0.01$, # compared with model control group, $p < 0.01$ and $ compared with western medicine group, $p < 0.01$.

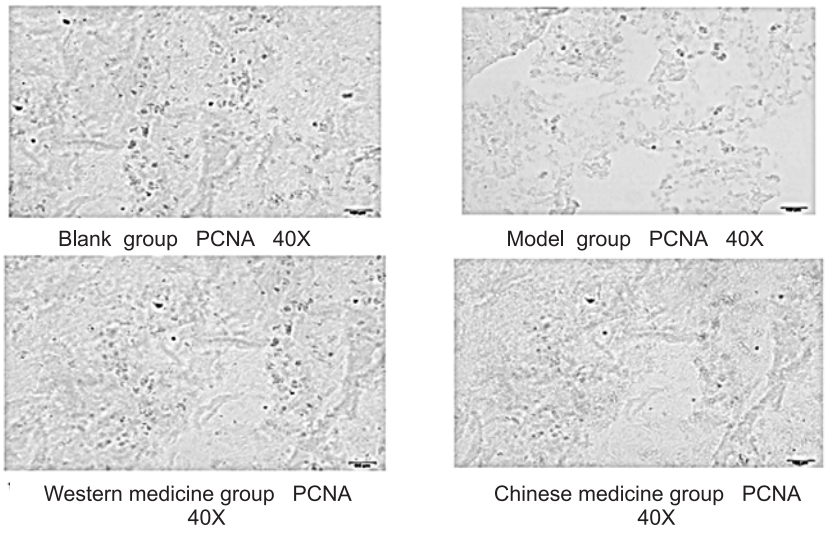


Figure 5. Immunohistochemical staining results of fibroblasts of rat's granulation tissue

rescence microscope (BX60, made by OLYMPUS, JAPAN) was used to observe the wound of tissue and pathomorphological characteristics of the surrounding tissue (12).

***Number of fibroblasts in granulation tissue and capillary expression test with PCNA and CD34 staining***

SP method of PCNA and CD34 immunohistochemistry staining include avoiding sections falling from glass slide by chemical reagent, dewaxing, inactivating endogenous enzymes, thermal remediation of antigen, dropwise adding PCNA (CD34) monoclonal antibody, DAB color developing, staining with hematoxylin, dehydrating, clearing, sealing sections and last microscopic observation (13).

**Statistical method**

SPSS version 19.0 is used to conduct ANOVA analysis of serological tests and pathological image analysis. Least significant difference (LSD) and Dunnett t-test were used for pairwise comparisons. In addition, $p < 0.05$ was set as significant difference.

## RESULTS

**Inflammation factors and growth factors**

Compared with the blank group, TNF-α, IL-1, hs-CRP in the serum of rats in the model controlled group go up significantly, and bFGF, EGF, VEGF and PDGF went down. During the advanced stage, AGES rises markedly and the difference is significant ($p < 0.01$). Compared with model control group, TNF-α, hs-CRP, IL-1 in the serum of rats in the western medicine and Chinese medicine groups went down markedly, bFGF, EGF, VEGF and PDGF went up markedly. During the advanced stage, AGES declines markedly and the difference is significant ($p < 0.01$). Compared with the western medicine group, TNF-α, IL-1 in the serum of rats in the Chinese medicine group went down clearly, the difference is significant ($p < 0.01$), FGF, EGF, PDGF, VEGF went up clearly, during the advanced stage, AGES declines clearly, while hs-CRP has no significant difference ($p > 0.05$) (Table 1).

**Microscopic observation of paraffin sections of granulation tissue of rats and hematoxylin and eosin (HE) staining**

There are a few of inflammatory cells, lots of fibroblasts, which are bigger cells with much cytoplasm, markedly colored nucleolus in the blank group, while the model group has more such inflammatory cells as neutrophils, lymphocytes and so on, less fibroblasts, some new capillary. In Chinese medicine group, there are a few of inflammatory cells, lots of fibroblasts, which are bigger cells with much cytoplasm, markedly colored nucleolus. The western medicine group has a few of inflammatory cells, more than the Chinese medicine group, less

fibroblasts than it, which are smaller cells, whose cytoplasm's protuberance was not obvious (Fig. 3).

**Immunohistochemistry**

Compared with the blank group, capillary density and fibroblast number in the model group are significantly ($p < 0.05$) decreased. Compared with the model group, the capillary density and fibroblast number in the Chinese and western group are significantly ($p < 0.05$) increased. Compared with the western medicine group, the capillary density and fibroblast number in the Chinese medicine group are significantly ($p < 0.05$) increased (Table 2 and Figs. 4, 5).

## DISCUSSION

**Influence of inflammatory factors on wound healing after diabetic ulcer**

Hyper-sensitive C-reactive protein (hs-CRP) is a type of non-specific inflammatory marker, produced by liver induced by proinfammatory cytokines (including IL-1, TNF-α) derived from endotheliocytes, macrophagocytes and adipocytes (6). Hs-CRP is a type of acute inflammatory factor, which is sensitive to inflammation reaction. When the wound surface appears, the increased level of hs-CRP facilitates leukocyte chemotaxis and platelet aggregation, contributing to the wound's anti-infection and hemostasis. However, in this disease the continuous high level of hs-CRP would be one of the factors leading to wound ischemia, not good for wound healing. Therefore, reducing hs-CRP content would put inflammation under effective control, and lower the rates of wound ischemia (7). Tumor necrosis factor-α (TNF-α) is produced mostly by monocytes and macrophagocytes, also can be secreted by some inflammation cells. It has various biological effects, such as strengthening phagocytic ability of neutrophils, inhibiting production of tumor cell and acting on liver, increasing the production of hs-CRP. The increase of TNF-α content would also lead to such side effects as infectious shock and cachexy. Interleukin 1 (IL-1) is mainly produced by mastocytes and monocytes. It could also be generated by neutrophils, epithelial cells, endothelial cells, B/T lymphocytes and vascular smooth muscle cells. IL-1 is involved in all kinds of inflammation phenomenon, metabolism and cytothesis. Besides, as endocrine hormone, IL-1 could stimulate whole body's inflammation reaction, making engine body into the stress state, and regulating the cells in immune system (8), closely related to wound healing. A large number of IL-1 could induce liver acute phase protein synthesis, triggering fever and cachexy, not good for wound healing.

Local inflammatory reaction would do certain harm to organization production, reducing the excessive synthesis and release of inflammation mediators such as hs-CRP/IL-1/TNF-α, helping to control inflammation reaction degree, and further helping wound healing.

According to this experiment's result, hs-CRP, TNF-α and IL-1 in rats' serum could decrease after medicine intervention, and in the Chinese medicine group the effect on decreasing TNF-α, IL-1 is obvious. Considering yinhuang powder, it could improve the inflammation condition of rats, which may be related to wound healing facilitation.

**Impact of growth factors on wound healing after diabetic ulcer**

Basic fibroblast growth factor (bFGF) is the major regulatory factor of tissue vascularization, which can promote the formation of new capillary. It is also the chemokinetic agent of fibroblast and a powerful growth stimulant. bFGF participates in several links during the process of wound healing. Previous studies have demonstrated that the expression of bFGF in local tissue of diabetic foot did not decrease (10). However, plenty of the bFGF lost their biological activity because of glycosylation. Therefore, wide glycosylation of bFGF protein without adequate new-born bFGF may be one of the essential causes of the difficult-to-heal diabetic foot ulcer wounds (10). Epidermal growth factor (EGF) is a polypeptide which can promote or restrain the growth of multiple kinds of cells. The combination of EGF receptor and EGF inside body produces receptor EFG compound. The compound regulates and controls important gene related to cell proliferation through a series of biochemical reactions, activates the process of wound healing and promotes the development of epithelial cells (11). In addition, EGF is beneficial to the proliferation and transportation of the vascular endothelial cell, as well as the formation of capillary. Vascular endothelial growth factor (VEGF) is a kind of active peptide which is secreted by macrophage, keratinocyte. It helps the proliferation of vascular endothelial cells and the combining of capillaries into bigger vessels. Moreover, VEGF can regulate the vascular endothelial cells proliferation and transport and promote the formation of new capillaries (11-15). During wound healing, together with FGF, VEGF stimulates endothelial cell growing and boosts the wound vascularization progress. Platelet derived growth factor (PDGF) is the serum growth factor which is released

by macrophage, keratinocyte, and stored in platelets. PDGF affects tissue repair cells, accelerates cells proliferation and boosts wound healing. In the advanced stage of wound healing, PDGF promotes the shrink of collagen matrix and condenses the wound. Besides, PDGF plays an important role in the remodeling stage.

In different processes of wound healing, growth factors play a role in various biological effects like chemotaxis, synthesis and secretion, proliferation and differentiation, apoptosis-inducing and angiogenesis, stimulating them in order to accelerate the wound healing. The imbalance of the types and content of endogenous growth factors of the wound, as well as the decline of the receptor activity, can lead to difficulty in wound healing (16).

According to this experiment's result, the level of bFGF, EGF, VEGF, PDGF in the rat serum can be improved after drug intervention. Yinhuang powder can remarkably enhance the level of bFGF, EGF, VEGF, PDGF in the rat serum, thus promoting the healing of diabetic ulcers.

**Impact of AGEs on wound healing after diabetic ulcer**

AGEs are irreversible end products. In normal bodies, amino acid and reducing sugar generate Amadori products that can produce highly active carbonyl compound through dehydration, oxidation and chemical rearrangement. Then, the compound reacts with free amino group and condenses into AGEs (16). Under the circumstances of continuous high sugar content, the aldehyde group of sugar and the amino of protein can generate AGEs. During glycosylation, the functional activity of growth factor declines, which influence the proliferation and transportation of repair cell. Glycosylation can lead to protein breakage. Glycosylation of sphingomyelin can cause the phagocyte to secrete protease, which results in demyelination disorders (14). Therefore, the accumulation and high sugar condition of AGEs can cause damage to capillaries and peripheral vessels, and cut down the expression of neurotrophic factor (2). It can be seen from the result of this experiment that drug intervention can reduce the amount of AGEs in the rat serum. Yinhuang powder plays a significant role in lowering the level of AGEs in the rat serum, thus promoting the healing of diabetic ulcers.

**Angiogenesis and fibroblast**

In the process of wound healing, the formation of granulation tissue, which has direct impact on wound healing and its prognosis, is very important. The essence of granulation tissue is abundant fibroblast and new-born capillaries. New-born capillaries improve the microcirculation in the wound, accelerate the metabolism, provide rich nutrient and oxygen which is necessary in the process of tissue repairing, and promote the wound healing (17, 18). After skin injury, fibroblasts around the wound will be activated to start metaplasia. Stress fiber with contractility appears in cytoplasm and facilitates the process of wound healing

The result of this experiment shows that both metronidazole-glucose injection and yinhuang powder can promote the growth and proliferation of new-born capillaries. Yinhuang powder has the most significant effect on improving the number density of new-born granulation tissue. However, in terms of fibroblasts, both metronidazole-glucose injection and yinhuang powder can increase the amount of fibroblasts. Yinhuang powder also has the most remarkable influence on improving the number of fibroblasts. Yinhuang powder is beneficial to the proliferation of fibroblast and new-born capillaries and the growth of granulation tissue around the wound, therefore, accelerates the wound healing.

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# *IN VITRO* CHARACTERIZATION AND ASSESSMENT OF COSMETIC POTENTIALS OF W/O EMULSION CREAM CONTAINING 2% *PROSOPIS CINERARIA* EXTRACT

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**Abstract:** In recent years, cosmetic research shifted towards natural products. Attempts are made to formulate stable topical preparations containing natural bioactive constituents but instability of topical emulsion is still a challenge for researchers. The aim of this study was to formulate stable W/O emulsion cream loaded with pre-concentrated 2% *Prosopis cineraria* extract and to explore its *in vitro* characteristics as compared to base (without extract). Moreover, stable W/O emulsion cream was applied to healthy human volunteers to assess its cosmetic potentials. The samples of base and formulation were placed at 8, 25, 40°C and 40°C at 75% RH (relative humidity) in different stability chambers. Stability parameters i.e., color, liquefaction, phase separation by centrifugation, conductivity, pH were monitored for 28 days for both base and formulation by using mechanical instruments. Healthy human volunteers (n = 11) were used for panel test. Results were analyzed by using SPSS 15.0. It was found that both base and formulation were stable with respect to color, liquefaction and phase separation with time. Furthermore, zero conductivity was found by all the samples of base and formulation kept at 8, 25, 40°C and 40°C at 75% RH in different stability chambers for 28 days. The pH of base and formulation kept at 8°C was 5.64 ± 12.6 and 5.54 ± 7.92, at 25°C was 5.77 ± 14.5 and 5.27 ± 13.5, at 40°C was 5.17 ± 16.1 and 5.13 ± 11.4 and at 40°C at 75% RH was 5.8 ± 7.3 and 4.92 ± 9.2, respectively. Statistically significant panel test results were obtained. It is concluded that the formulation W/O emulsion cream is stable with respect to *in vitro* evaluation and results of panel test coined that it can be used cosmetically.

**Keywords:** W/O emulsion cream, *in vitro* characterization, pH, panel test

Emulsions are metastable colloids made out of two immiscible fluids, one being dispersed in other, in the presence of surface active agents. They are obtained by shearing two immiscible fluids leading to the fragmentation of one phase into the other. They are thermodynamically unstable and revert back to separate oil and water phases until kinetically stabilized by third component, the emulsifying agent. Because the shelf life of emulsions may become significant (more than a year), they become good candidates for various commercial applications (1).

In cosmetology, the quality of a product is determined by high merit of its stability (2). Stability testing of cream is one of the most important quality control measures of topical preparations. Shelf lives, temperature, humidity, sunlight, shipment, abrasion are some keen factors which directly affect the stability of creams (3). Water-in-oil emulsion systems do have desirable properties. However, one drawback to the use of such systems for commercial products is the difficulty associated with maintaining such systems stable against separation. World consumers are now focused on their health and well-being more than before. Different words such as ‘natural’, ‘organic’, ‘no artificial preservatives’ and ‘no animal ingredients’ are drawing alarming attention. This trend increase the demand

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for products formulated as cosmeceuticals with natural and nutraceutical ingredients. Active ingredients and new delivery systems are driving the new product development ground (4).

According to these trends, researchers struggle to develop highly differentiated products with center of treatment as well as aesthetics. A significant number of novel products are based on new natural bioactive ingredients. With these promising actives, come a variety of formulation challenges that includes stability control and the complications of combining several actives into a sole cosmetic product (5). The present study aimed to formulate stable emulsion cream containing natural plant extract and to ensure its aesthetic potentials.

## MATERIALS AND METHODS

The dried bark of plant (*Prosopis cineraria*) was collected from a field owned by Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Bahawalpur, Pakistan. The identification of dried bark was done at CIDS and voucher no. was 125/.I.U.2012. Paraffin oil was of Merck KGaA Darmstadt, Germany, emulsifying agent ABIL® EM 90 (Cetyl-PEG/PPG-10/1 Dimethicone) was purchased from Franken Chemicals, Gebinde, Germany and 70% ethanol was from Merck KGaA Darmstadt, Germany. The bees wax and deionized water was obtained from Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur Pakistan.

### Instruments

Centrifuge machine (Hettich EBA 20, Germany), cold incubator (Sanyo MIR-153, Japan), conductivity-meter (WTW COND-197i, Germany), digital humidity meter (TES Electronic Corp., Taiwan), electrical weight balance (Precisa BJ-210, Switzerland), homogenizer (Euro-Star, IKA D 230, Germany), hot incubator (Sanyo MIR-162, Japan), pH-Meter (WTW pH-197i, Germany), rotary evaporator (Eyela, Co. Ltd., Japan), refrigerator (Orient, Pakistan), water bath (HH .$S_{21\ 4}$, China) and SPSS 15.0 was used for statistical analysis.

### Preparation of formulation

The oily phase used in the preparation of formulation comprises of paraffin oil, bees wax, cetyl PEG/PPG-10/1 Dimethicone with HLB value 4-6 and heated up to75 ± 1°C. Meanwhile, aqueous phase (distilled water) was also heated up to 75 ± 1°C. The 2% preconcentrated *Prosopis cineraria* bark extract was added in the aqueous phase. After heating, aqueous phase was added to the oily phase drop by drop. Stirring was continued at 2000 rpm by the mechanical mixer for 15 min until all aqueous phase was added; 2 to 3 drops of rose water were added during this stirring time to give good fragrance to the cream. After complete addition of aqueous phase, speed of mixer was reduced to 1000 rpm for homogenization, for a period of 5 min and then speed of mixer was reduced to 500 rpm for further 5 min for complete homogenization; until cooled to room temperature.

### Preparation of base

The procedure adopted for the preparation of base was the same as for formulation except that no extract was incorporated in aqueous phase of base cream (Table 1).

### Participants

Eleven human volunteers were chosen whose ages were between 25 and 40 years. Only male volunteers were included in this study. Volunteers were examined for any serious skin disease or damage especially on cheeks and forearms. Every volunteer was provided with a volunteer protocol before continuation of study. Every volunteer signed the terms and conditions of the protocol for testing individually.

Table 1. Composition of emulsion (creams).

| Emulsion phase | Ingredients | Emulsion base (g) | Extract emulsion (g) |
|---|---|---|---|
| Oily | Paraffin oil | 14 | 14 |
| | Cetyl-PEG/PPG-10/1 Dimethicone | 3.5 | 3.5 |
| | Bees wax | 2 | 5 |
| Aqueous | Extract | —— | 2 |
| | Distilled water | 100 | 100 |

Table 2. Stability characteristics of base and formulation at 8°, 25°, 40°C and 40°C ± 75% RH.

| | | Fresh | | After 12 h | | After 24 h | | After 7 days | | After 14 days | | After 21 days | | After 28 days | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **B** | **F** | **B** | **F** | **B** | **F** | **B** | **F** | **B** | **F** | **B** | **F** | **B** | **F** |
| Color | **A** | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP |
| | **B** | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP |
| | **C** | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP |
| | **D** | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP | W | LP |
| Liquefaction | **A** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| | **B** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| | **C** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| | **D** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | +ve |
| Phase separation | **A** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| | **B** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| | **C** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| | **D** | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve | -ve |
| Conductivity | **A** | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | **B** | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | **C** | N | N | N | N | N | N | N | N | N | N | N | N | N | N |
| | **D** | N | N | N | N | N | N | N | N | N | N | N | N | N | N |

**B** = base, **F** = formulation, LP = light pink, W = white, -ve = no change, +ve = little change, N = zero electrical conductivity; **A** = at 8°C; **B** = at 25°C; **C** = at 40°C; **D** = at 40°C + 75% RH (RH = relative humidity).

**Ethical approval**

This study was approved by the Board of Advanced Study and Research (BASR), The Islamia University of Bahawalpur, and the institutional ethical committee, Department of Pharmacy, The Islamia University of Bahawalpur, Bahawalpur, Pakistan, in compliance with NIH Principles of Laboratory Animal Care, 1985. The reference no. is Pharm 1991/2013.

## RRSULTS AND DISCUSSION

### *In vitro* evaluation of W/O emulsion creams

For *in vitro* evaluation of both creams, formulation (having *Prosopis cineraria* extract) and base (without extract) were placed at different temperatures i.e., at 8, 25 and 40°C and 40°C at 75% RH (relative humidity) in stability chambers for 28 days (Table 2). No change in color, liquefaction and phase separation was observed; furthermore, the electrical conductivity test was also negative for each sample of creams. The samples of formulation evaluated at 8, 25 and 40°C and 40°C at 75% RH after of study period are shown in Figure 1.

In this study, the light pink color of the formulation was due to the presence of significant amount of tannins and tryptamines in the extract of plant. In formulation extracts were used as active ingredient and it is a rich source of natural antioxidants. These antioxidants have a potential of natural preserving

Table 3. pH value (mean ± standard error of mean) at different storage conditions of temperature.

| | 8°C | 25°C | 40°C | 40°C ± 75% RH |
|---|---|---|---|---|
| F | 5.54 ± 7.92 | 5.27 ± 13.5 | 5.13 ± 11.4 | 4.92 ± 9.2 |
| B | 5.64 ± 12.6 | 5.77 ± 14.5 | 5.17 ± 16.1 | 5.8 ± 7.3 |

F = formulation, B = base, RH = relative humidity

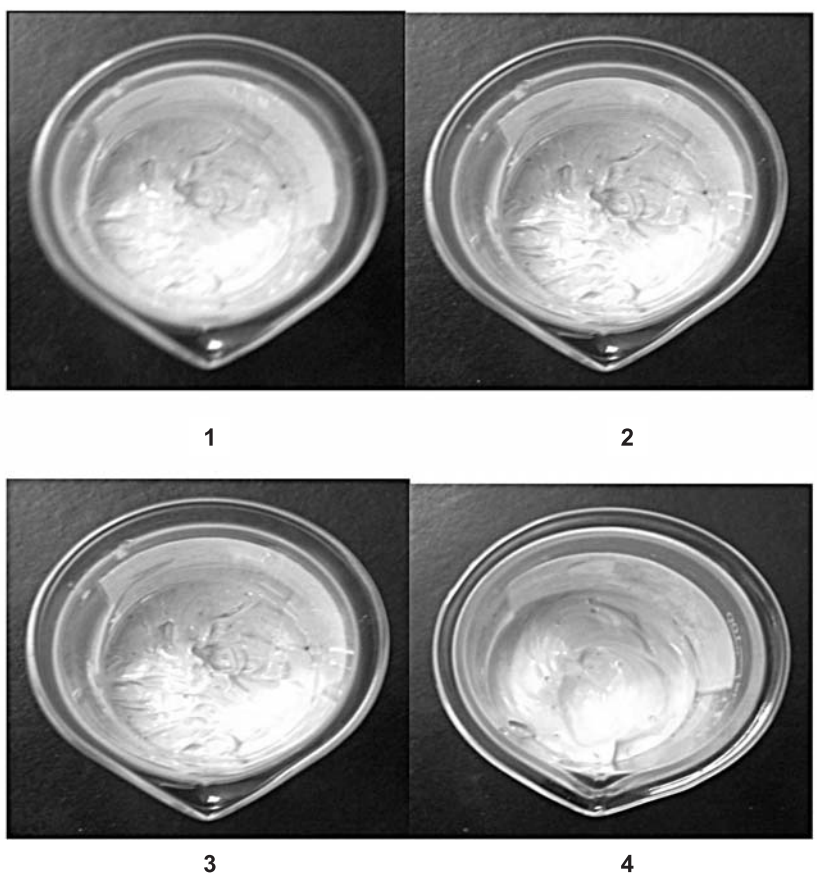


Figure 1. Samples of formulation. **1** = 8°C, **2** = 25°C, **3** = 40°C, **4** = 40°C ± 75% RH

Table 4. Panel test for base and formulation.

| | Average points for base ± SEM | Average points for formulation ± SEM |
|---|---|---|
| Ease of application | 4.21 ± 0.004 | 4.86 ± 0.003 |
| Sense just after application | 3.22 ± 0.004 | 4.02 ± 0.003 |
| Sense in long term | 3.68 ± 0.005 | 3.98 ± 0.005 |
| Spreadability | 4.01 ± 0.003 | 5.02 ± 0.004 |
| Irritation | 0.00 ± 0.000 | 0.00 ± 0.000 |
| Shine on skin | 3.47 ± 0.006 | 3.22 ± 0.006 |
| Sense of softness | 4.42 ± 0.005 | 4.54 ± 0.004 |

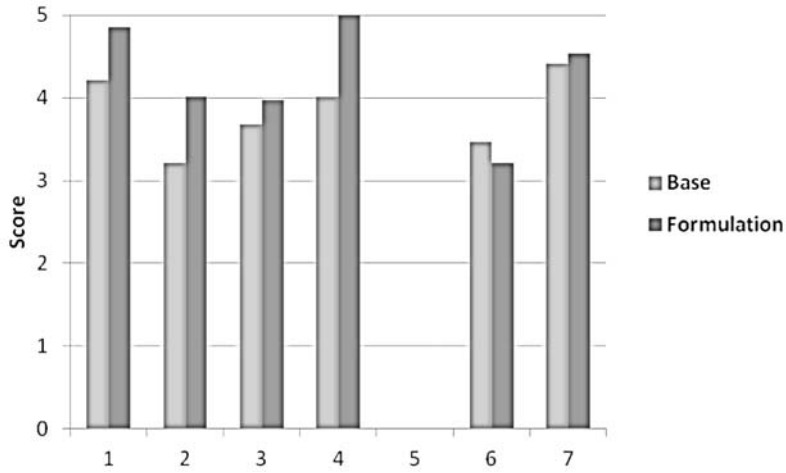


Figure 2. Average values for panel test. 1 = Ease of application, 2 = Sense just after application, 3 = Sense in long term, 4 = Spreadability, 5 = Irritation, 6 = Shine on skin, 7 = Sense of softness

and may protect the cream from microbial growth and of oxidative degradation, which may change the color of cream during shelf life. Many other factors support color stability of formulation and base such as Cetyl PEG/PPG-10/1 Dimethicone -a colorless, non toxic and clear liquid emulsifying agent (6). It acts at interphase of dispersed and continuous phase and properties of emulsion cream heavily depend on its nature and type. Bees wax is naturally produced by bees and used for a long time to control the change in color of creams, paraffin oil is a blend of purified liquid hydrocarbons and it controls the viscosity and stability of creams. Stock's Law reveals the fact that increasing the viscosity of the continuous phase enhances the shelf life of topical preparations (7).

**pH test**

Skin pH is an important indicator of topical preparation stability especially in case of creams (2). The average pH of human skin ranges from 5.5 to 6. Hence, the pH of topical preparations must be in accordance with skin's pH (7). The change in pH values of both base and formulation kept at 8, 25 and 40°C and 40°C ± 75% RH have been evaluated**.**

The pH of base and formulation at different storage conditions of temperature and humidity were in the range of normal skin pH. The pH of freshly prepared base and formulation was 6.66 and 6.64, respectively. The samples of base and formulation showed gradual decrease in pH from 12 h to 28$^{th}$ day study period. At the end of study (on 28$^{th}$ day) pH of base samples decreased to 5.64 ± 12.6, 5.77 ± 14.5, 5.17 ± 16.1, 5.8 ± 7.3, while in case of formulation pH decreased to 5.54 ± 7.92, 5.27 ± 13.5, 5.13 ± 11.4, and 4.92 ± 9.2, respectively, as shown in Table 3. When the results were manipulated by statistical technique ANOVA at 5% level of significance, in base samples of different temperature; it was found that the change in pH was

insignificant at different time intervals. In case of formulation samples which were placed at different temperatures, the same insignificant results were seen. The paraffin oil produces aldehyde and different organic acids on oxidation at accelerated temperature. This may be a reason in lowering of pH of base and formulation samples (6). Furthermore, Naveed et al. described that at different storage conditions of temperature, the ingredient of both creams decomposed (8). It has been reported that the bark of *Prosopis cineraria* (L.) Druce have significant amount of gallic acid (9), palmitic acid, stearic acid, oleic acid and linoleic acid (10). This significant change in decrease in the pH of formulation with respect to base is due to these acidic secondary metabolites of *Prosopis cineraria* (L.) Druce.

**Panel test**

The results of panel test showed that there were no difficulty in applying the creams by volunteers, the sense in long term of formulation was greater than base and formulation produces more pleasant feeling than base by applying on skin. It showed that sense just after the application of formulation was more pleasant and has greater spreadability. No skin irritation was produced by any of the creams on volunteer's skin.

The results of panel tests of both base and formulation by volunteers have been presented in Table 4 and Figure 2.

The shine on skin with base was a little bit greater than that with formulation. This was due to more absorption value and spreadability of the formulation than base. The softness of skin by formulation was greater than base. Softness may be produced by paraffin oil which was present in the same quantity in both formulation but formulation containing extract of *Prosopis cineraria* have high amount of polyphenols which increases blood supply to skin (11).

## CONCLUSION

It is concluded that the formulation cream is stable with respect to all *in vitro* characterization. There is no color change, no liquefaction, and no phase separation throughout the study period. Beside this, the pH of formulation cream is in accordance with normal skin pH range so it can be used without any skin reaction. The results of panel test clears that formulation cream has marvelous characteristics to be used cosmetically. Further *in vivo* research is needed to evaluate formulation cream for its cosmetic effects on human skin.

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**Conflict of interest**

The authors declare no conflict of interest in this study.

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# ANTIOXIDANT ACTIVITY OF *GEUM RIVALE L.* AND *GEUM URBANUM L.*

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**Abstract:** Two species of the Rosaceae genus *Geum*, comprising high amounts of tannins and phenolic acids, were investigated in terms of their antioxidant activity. Antioxidant potential of different extracts from aerial and underground parts of *Geum rivale* and *G. urbanum* was studied using various *in vitro* methods (FC, DPPH, FRAP and linoleic acid peroxidation test). The hydromethanolic extract from the roots of *G. rivale* with high total phenolic content of 17.48% was proven to be about twice as effective as the other extracts tested. Ethyl acetate and *n*-butanol were demonstrated to be the best solvents for optimal concentration of antioxidant constituents.

**Keywords:** *Geum*, Rosaceae, DPPH, FRAP, linoleic acid peroxidation

Reactive oxygen species (ROS) are highly reactive chemical molecules containing an atom of oxygen. They are constantly produced in every aerobic cell during the normal metabolic processes and most of them are quickly scavenged by cellular antioxidant systems consisting of enzymatic and non-enzymatic antioxidants. This natural mechanism, however effective, can sometimes fail, especially when the formation of ROS is enhanced by harmful factors like some chemicals, UV radiation or inflammatory processes. The resulting imbalance leads to the state known as "oxidative stress", during which the excessive ROS can damage cell structures, inhibit enzymes or oxidize nucleic acids. The destructive power of this phenomenon is widely acknowledged, and it is believed to be the cause of many diseases such as atherosclerosis, neurodegenerative diseases or cancer, as well as accelerated ageing (1).

In order to help maintain the fragile balance between the production and the destruction of the ROS, exogenous antioxidants can be applied, and plants area is considered to be a good source of natural, safe compounds with a large antioxidant potential (2-4).

*G. rivale* and *G. urbanum* are perennial herbs belonging to the Rosaceae family, widely distributed across the Europe, and commonly found either in forests and parks (*G. urbanum*) or on wet meadows and river banks (*G. rivale*) (5, 6). They are valued in folk medicine for their astringent and antiseptic properties, and scientific inquiries, motivated by the traditional use, have revealed the presence of tannins, phenolic acids, triterpenes, flavonoids and essential oil, in aerial and underground parts of the plants alike (7-9). Our investigations concerning the chemical composition of the two species showed, that particularly tannins and phenolic acids are present in significant quantities, both groups of compounds belonging to the wide class of polyphenols, that are known to play an important role in the antioxidant activity of plant materials.

There are only a few studies on the antioxidant activity of the plants from the genus *Geum*. The activity of methanolic extract from the roots of *G. rivale* was studied by Oszmianski et al. (10), using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] assays. The results proved, that the extract was three times more effective in scavenging free radicals than extracts from underground organs of two other Rosaceae species – *Filipendula ulmaria* and *Aruncus silvestris*. On the other hand, its activity was weaker than the activity of extracts from the roots of *Potentilla alba* and *Waldstenia geoides* but only by a factor of 1.5. The antioxidant potential of *G. urbanum* was, in turn, investigated by Mantle et al. (11). The hydromethanolic extract form the leaves of the plant, tested with ABTS assay, was demonstrated to have higher activity than 37 out of 38 other extracts evaluated.

Above results, although promising, give only a partial insight into the problem. Because of different

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methodology used, their results are difficult to compare, and, as both plants are used in traditional medicine to some extent interchangeably, their direct comparison would definitely be of some interest. Moreover, there is no information about the best solvent to concentrate the antioxidant constituents of the plants. The aim of our study was, therefore, better characterization of the antioxidant potential of aerial and underground parts of *G. rivale* and *G. urbanum*, by assessing the activity of extracts of different polarities using various *in vitro* tests.

## EXPERIMENTAL

### Plant material

Aerial and underground parts of wild growing *G. rivale* and *G. urbanum* were collected from locations in Lódź during the flowering of the plants. The material was identified by Prof. Jan Gudej, Department of Pharmacognosy, Medical University of Lodz, Poland. Voucher specimens were deposited in Department of Pharmacognosy, Medical University of Lodz, Poland.

Plant material was dried under normal conditions, powdered with electric grinder and sieved through a 0.315 mm sieve.

### Chemicals and instrumentation

Chromatographic grade purity reagents and standards: 2,2-diphenyl-1-picryl hydrazyl (DPPH), 2,2'-azobis-(2-amidinopropane) dihydrochloride (AAPH), 2,4,6,-tris-(2-pyridyl)-s-triazine (TPTZ), linolenic acid and (±)-6-hydroxy-2,2,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were obtained from Sigma-Aldrich (Germany/USA). All other chemicals and solvents of analytical grade were purchased from POCh (Poland).

Samples were incubated in a constant temperature using a BD 23 incubator (Binder, Germany). Absorbance was measured using a Lambda 25 spectrophotometer (Perkin-Elmer, USA), in 10 mm quartz cuvettes.

### Preparation of hydromethanolic extracts

Samples of plant material (100 mg or 1 g depending on the assay) were refluxed with 30 mL of 70% methanol for 30 min, and then twice with 20 mL of 70% methanol for 20 min. Combined extracts were filled up to 100 mL and left for further analysis (hydromethanolic extract – HME).

### Preparation of dried extracts and fractions

Samples of plant material (30 g) were first exhaustively extracted with petroleum ether (250 mL) and then with chloroform (250 mL) in a Soxhlet apparatus. Subsequently, they were refluxed trice with 100 mL of methanol. The methanol extract was evaporated to dryness *in vacuo* (dry methanolic extract – DME, 4.5-5.3 g depending on the plant material). A part of DME (1 g) was left to further analysis and the rest was suspended in water and extracted successively with diethyl ether, ethyl acetate and *n*-butanol. The fractions were evaporated and left for further analysis (diethyl ether fraction – DEF, 0.2-0.5 g; ethyl acetate fraction – EAF, 0.7-1.1 g; *n*-butanol fraction – BF, 1.8-2.3 g).

### Determination of total phenolic content (TPC)

The amount of total phenolics was determined according to the Folin-Ciocalteu (FC) method (12) with the use of HME (prepared from 100 mg of dry plant material) or methanolic solutions of DME, DEF, EAF and BF (60-200 μg/mL). Results were calculated with the use of eight-point calibration curve of gallic acid and expressed as gallic acid equivalents (GAE) per dry weight of the plant material or extract/fraction.

### DPPH free radical-scavenging test

The scavenging activity was determined based on the method of Brand-Williams, Cuvelier, and Berset (13) with slight modifications as described previously (14). The range of concentrations used was 30-120 μg/mL for HME and 5-40 μg/mL for DME and fractions thereof. The activity of samples was expressed as $EC_{50}$ value which is the concentration of the sample that reduces the amount of DPPH radicals by 50%. To improve accuracy of the measurements we used, similarly as in our previous paper, normalized $EC_{50}$ values, that are not influenced by slight differences in the initial condition of the reaction.

### Ferric reducing antioxidant power (FRAP) assay

The FRAP was determined according to the method of Pulido et al. (15), with some variations described previously (12). The concentration used was 30 μg/mL for HME and 1.9-4.0 μg/mL for DME and fractions thereof. The antioxidant activity was expressed in micromoles of ferrous ions produced by 1 g of the dry extract, fraction or standard, which was calculated from the eight-point calibration curve of ferrous sulfate.

### Linoleic acid (LA) peroxidation test

The ability of the analytes to inhibit AAPH-induced LA peroxidation was assayed according to the method of Azuma et al. (16) with some modifi-

cations described previously (14). The range of concentrations used was 1.25-10.0 mg/mL for HME and 0.3-6 mg/mL for DME and fractions thereof. The activity of the analytes was expressed as $IC_{50}$, which is the concentration of the sample that decreases the degree of LA oxidation by 50%.

**Statistical analysis**

The samples of each analyte (extract, fraction or standard) were analyzed for LA-peroxidation test in triplicate and data are reported as the mean (n = 3 × 1) ± SD (standard deviation). For other photometric methods, three samples of each analyte were assayed, each sample was analyzed in quintuplicate and data are reported as the mean (n = 3 × 5 × 1) ± SD. The statistics (calculation of SD, one-way analysis of variance, Duncan's tests, and linearity studies) were performed using the software Statistica PL for Windows (StatSoft Inc., Poland).

## RESULTS AND DISCUSSION

A variety of methods have been developed to evaluate the antioxidant potential of plant materials. The underlying reactions can proceed according to two main mechanisms. Single electron transfer (SET) reaction mechanism is connected with the transfer of a single electron from the molecule of the antioxidant to other molecule or ion, resulting in its reduction. In hydrogen atom transfer (HAT) dependent reaction the same effect is caused by the transfer of a hydrogen atom (17, 18). In our studies we used SET as well as HAT-based methods, in order to characterize different aspects of the antioxidant activity of the samples.

The DPPH assay is a discoloration test, in which the ability of the extract to scavenge the DPPH radical is measured. It is based largely on SET reaction mechanism, with hydrogen atom transfer playing only a marginal role. It is a simple and fast assessment, extensively used in many research laboratories, often as a preliminary method for estimating the antioxidant activity of natural products. DPPH is however, a synthetic radical that bears no similarity to free radicals occurring in living cells (13, 17).

The FRAP assay is a method, that depends entirely on SET reaction mechanism. It measures the ability of the sample to reduce $Fe^{3+}$ to $Fe^{2+}$, and as such, also bears no relation to physiological conditions. It still permits, however, to assess the reductive power of the compounds tested and reflect their capacity to modulate redox processes in general (15, 17).

The principle of FC assay is a redox reaction proceeding mostly *via* SET mechanism, and for that reason the method could be considered as an another measure of the antioxidant activity. However, it is also a useful procedure for estimating the total phenolic content in the sample (17). It is also the only assay among the selected ones, the results of which due to the standardized conditions are easily comparable with the literature data.

LA peroxidation test, as opposed to other methods described, depends fully on the HAT reaction. It models more accurately physiological conditions, as it evaluates the scavenging potential of the tested compounds towards peroxy radicals, that are one of the naturally occurring ROS. It also provides insight into the ability of the sample to prevent harmful effects caused by the free radicals (in this case peroxidation of LA) (17-19).

The selected methods described above were used to test the antioxidant activity of hydromethanolic extracts (HMEs) of aerial and underground parts of *G. rivale* and *G. urbanum*, as well as the antioxidant activity of dry methanolic extracts (DMEs) and fractions obtained from DMEs by extraction with solvents of different polarities.

**Hydromethanolic extracts**

The results of the determination of the antioxidant activity and the total phenolic content for

Table 1. Antioxidant activity and total phenolic content of HMEs.

| Plant material | | DPPH | FRAP | LA oxidation inhibition | TCP |
|---|---|---|---|---|---|
| | | $EC_{50}$ [μg/mL] | FRAP [mM/g] | $IC_{50}$ [μg/mL] | [%] GAE |
| *G. rivale* | Aerial parts | 23.97 ± 0.85$^{b}$ | 2.29 ± 0.10$^{b}$ | 413.47 ± 20.54$^{c}$ | 7.83 ± 0.11$^{a}$ |
| | Underground parts | 11.22 ± 0.36$^{a}$ | 3.84 ± 0.14$^{c}$ | 252.84 ± 11.32$^{a}$ | 17.48 ± 0.46$^{b}$ |
| *G. urbanum* | Aerial parts | 26.57 ± 1.24$^{c}$ | 1.89 ± 0.07$^{a}$ | 366.60 ± 14.47$^{b}$ | 7.61 ± 0.36$^{a}$ |
| | Underground parts | 26.92 ± 1.31$^{c}$ | 1.94 ± 0.06$^{a}$ | 544.74 ± 26.43$^{d}$ | 7.89 ± 0.27$^{a}$ |

Different superscripts in each column indicate significant differences in the mean values at $p < 0.05$.

HMEs are shown in Table 1. Among the HMEs tested, the extract from the underground parts of *G. rivale* exhibited the highest activity regardless of the method used. Its $EC_{50}$ value in DPPH assay was 11.22 μg/mL, while the FRAP value was 3.84 mM/g. Other HMEs with $EC_{50}$ in range 23.97-26.92 μg/mL and FRAP in range 1.89-2.29 mM/g had about two times weaker activity.

The $IC_{50}$ values for the inhibition of LA peroxidation varied from 544.74 to 252.84 μg/mL. The HME from the rhizomes of *G. rivale* was still the most active extract, while the HME from the rhizomes of *G. urbanum* was the least active one.

The results of FC total phenolic assay were in agreement with above findings showing almost twice higher content of phenolic compounds in the underground parts of *G. rivale* (17.48% GAE) comparing with other plant material tested (7.61-7.89% GAE). They also correspond with our previous studies that showed much higher content of tannins and phenolic acids in the rhizomes of *G. rivale* (20, 21). These two groups of compounds have, therefore, probably the greatest significance in the antioxidant activity of investigated *Geum* tissues.

High TPC values are not uncommon for plant materials acquired from species of Rosaceae family. In the studies by Cai et al. (22) evaluating 112 Chinese herbs, the roots of *Sanguisorba officinalis* and flowers of *Rosa chinensis* were among the ones containing the highest amounts of phenolic compounds, with TPC values of 15.87% GAE and 18.75% GAE, respectively. These results were comparable to those obtained for the leaves of *Camellia sinensis* (TPC = 17.40% GAE) and fruits of *Punica granatum* (TPC = 22.56% GAE), that are recognized as a valuable source of phenolics. The high values of TPC were also reported for the inflorescences and leaves of selected *Sorbus* species (TPC = 6.06-11.83% GAE) tested in one of our previous studies (12). The research conducted by Buricova et al. (23) revealed as well, significant amounts of polyphenols in the leaves of *Fragaria vesca* (TPC = 6.24% GAE), *Rubus idaeus* (TPC = 6.89% GAE) and *Rubus fruticosus* (TCP = 7.54% GAE). The investigated *Geum* species are, therefore, another Rosaceae plants, which can be considered as a good source of polyphenol rich plant materials.

Table 2. Antioxidant activity and total phenolic content of DMEs and fractions thereof.

| Plant material | | Extract/ Fraction | DPPH | FRAP | LA oxidation inhibition | TPC |
|---|---|---|---|---|---|---|
| | | | $EC_{50}$ [μg/mL] | FRAP [mM/g] | $IC_{50}$ [μg/mL] | [%] GAE |
| ***G. rivale*** | Aerial parts | DME | $12.47 \pm 0.54^{h}$ | $3.46 \pm 0.16^{a}$ | $176.54 \pm 8.34^{j}$ | $19.46 \pm 1.16^{a}$ |
| | | DEF | $6.01 \pm 0.17^{f}$ | $9.40 \pm 0.39^{c}$ | $67.49 \pm 3.27^{d}$ | $49.83 \pm 1.10^{e}$ |
| | | EAF | $2.92 \pm 0.13^{a}$ | $17.62 \pm 0.68^{jk}$ | $49.57 \pm 2.21^{b}$ | $63.05 \pm 0.39^{h}$ |
| | | BF | $4.11 \pm 0.18^{b}$ | $12.41 \pm 0.54^{ef}$ | $70.80 \pm 3.06^{d}$ | $46.49 \pm 2.35^{cd}$ |
| | Underground parts | DME | $4.22 \pm 0.15^{bcd}$ | $13.00 \pm 0.44^{f}$ | $129.30 \pm 5.97^{i}$ | $75.39 \pm 2.57^{j}$ |
| | | DEF | $4.62 \pm 0.21^{d}$ | $12.26 \pm 0.57^{ef}$ | $89.88 \pm 4.15^{f}$ | $63.43 \pm 2.17^{h}$ |
| | | EAF | $2.99 \pm 0.09^{a}$ | $18.56 \pm 0.62^{l}$ | $59.16 \pm 2.37^{c}$ | $65.59 \pm 0.76^{hi}$ |
| | | BF | $3.35 \pm 0.15^{a}$ | $15.79 \pm 0.63^{h}$ | $59.04 \pm 2.29^{c}$ | $82.41 \pm 2.73^{k}$ |
| ***G. urbanum*** | Aerial parts | DME | $13.34 \pm 0.47^{i}$ | $3.07 \pm 0.11^{a}$ | $189.22 \pm 8.74^{k}$ | $19.06 \pm 0.91^{a}$ |
| | | DEF | $6.44 \pm 0.23^{g}$ | $6.29 \pm 0.28^{b}$ | $119.27 \pm 5.67^{h}$ | $38.55 \pm 1.02^{b}$ |
| | | EAF | $4.18 \pm 0.11^{bc}$ | $14.07 \pm 0.67^{g}$ | $72.28 \pm 3.48^{d}$ | $53.17 \pm 2.61^{f}$ |
| | | BF | $6.09 \pm 0.26^{fg}$ | $12.05 \pm 0.38^{e}$ | $73.91 \pm 3.12^{de}$ | $44.37 \pm 1.83^{c}$ |
| | Underground parts | DME | $4.57 \pm 0.20^{cd}$ | $10.42 \pm 0.43^{d}$ | $92.03 \pm 4.51^{f}$ | $48.53 \pm 1.25^{de}$ |
| | | DEF | $5.56 \pm 0.22^{e}$ | $9.29 \pm 0.37^{c}$ | $74.53 \pm 3.49^{de}$ | $48.28 \pm 1.20^{de}$ |
| | | EAF | $3.16 \pm 0.07^{a}$ | $16.87 \pm 0.65^{ij}$ | $68.05 \pm 3.11^{d}$ | $57.11 \pm 2.50^{g}$ |
| | | BF | $3.32 \pm 0.13^{a}$ | $16.28 \pm 0.72^{hi}$ | $80.96 \pm 3.79^{e}$ | $67.13 \pm 1.33^{i}$ |
| Trolox | | | $3.27 \pm 0.10^{a}$ | $9.42 \pm 0.31^{c}$ | $22.45 \pm 1.10^{a}$ | - |

Different superscripts in each column indicate significant differences in the mean values at $p < 0.05$.

**Dry methanolic extracts and fractions thereof**

The results of the determination of the antioxidant activity and the total phenolic content for DMEs and the fractions are shown in Table 2.

The normalized $EC_{50}$ values for DPPH assay were in range 2.92-13.34 μg/mL. In case of DMEs, the extracts from the underground parts of the plants had significantly lower $EC_{50}$ values (4.22-4.57 μg/mL) than the extracts from the aerial parts (12.47-13.34 μg/mL). Taking into consideration fractions of different polarities, EAFs were the most active ones ($EC_{50}$ = 2.92-4.18 μg/mL), followed by BFs ($EC_{50}$ = 3.32-6.09 μg/mL). The least active fractions, regardless of the plant material tested, were DEFs ($EC_{50}$ = 4.62-6.44 μg/mL). The activity of the most active fractions with $EC_{50}$ ranging from 2.92 to 3.35 μg/mL was similar to the activity of the Trolox standard ($EC_{50}$ = 3.27 μg/mL) with no statistically significant differences ($p < 0.05$).

The FRAP values varied from 3.07 to 18.56 mM/g and corresponded largely to the $EC_{50}$ values from DPPH assay, which was confirmed by significant correlation ($r = 0.8443$, $p < 0.05$) between the two variables. DMEs from the rhizomes of the plants (FRAP = 10.42-13.00 mM/g) were again over three times more active than DMEs from the aerial parts and the order of antioxidant potential of fractions was parallel to that of DPPH assay with AEFs being the most active ones, followed by BFs and DEFs. The most active fractions – EAFs and BFs (FRAP = 12.05-18.56 mM/g) had substantially higher reduction potential than Trolox standard (FRAP = 9.42 mM/g).

The $IC_{50}$ values in LA peroxidation test ranged from 49.57 μg/mL to 189.22 μg/mL. The results of the test were significantly correlated with the results of the DPPH assay ($r = 0.8691$, $p < 0.05$) and also to some extent to the FRAP assay ($r = 0.6815$, $p < 0.5$). The DMEs from the underground parts ($IC_{50}$ = 92.03-129.30 μg/mL) of the plants were still more active than the DMEs from the aerial parts ($IC_{50}$ = 176.54-189.22 μg/mL), but the differences were not as substantial as in the previous tests. In the case of the aerial parts of *G. rivale* and the rhizomes of *G. urbanum*, EAFs and BFs were more active than DEFs, whereas in the case of the underground parts of *G. urbanum*, all fractions exhibited similar activity. As far as the aerial parts of *G. rivale* are concerned, the AEF was the strongest inhibitor of LA peroxidation. With $EC_{50}$ = 49.57 μg/mL it was also the most active fraction of all fractions tested, but still over two times weaker than Trolox standard ($IC_{50}$ = 22.45 μg/mL).

The TPC determined by FC assay varied from about 19% GAE in the DMEs from the aerial parts of the plants to about 80% in BF from the underground parts of *G. rivale*. EAFs were the fractions containing the highest amounts of polyphenols in the case of the aerial parts, and BFs in the case of the underground parts. The TPC results were significantly correlated with the results of DPPH ($r = -0.8523$, $p < 0.05$) and FRAP ($r = 0.8442$, $p < 0.05$) assay. The correlation was weaker, although still significant, in the case of LA peroxidation test ($r = -0.6649$, $p < 0.05$).

In general, ethyl acetate and *n*-butanol seem to be the best solvents to concentrate antioxidants from methanolic extracts of the investigated *Geum* species. Similar results were obtained in our previous studies concerning selected plants of the genus *Sorbus*, where EAFs and BF were the most active fractions in FC, DPPH, ABTS and FRAP assays (14). Moreover, in both cases strong correlations were identified between TPC values and SET-based antioxidant capacity. The findings varied, however, when it comes to HAT-type activity. The significant correlation between TPC values and the ability of the extracts to inhibit LA peroxidation was found for *Geum* species, while there was no clear correspondence between $IC_{50}$ values of LA peroxidation test and total phenolic content in evaluated *Sorbus* extracts (14). The fact that the both genera vary in terms of their chemical composition could be probably accounted for these results.

## CONCLUSION

The determination of the antioxidant activity of HMEs from aerial and underground parts of *G. rivale* and *G. urbanum* led to the identification the rhizomes of *G. rivale* as the plant material with the highest antioxidant potential. Significant correlations between TPC values and the antioxidant capacity evaluated by DPPH, FRAP and LA peroxidation assays suggest that, in the case of *Geum* extracts, polyphenols are mainly responsible for both SET and HAT-type antioxidant activity. Furthermore, ethyl acetate and *n*-butanol were proven to be the most efficient solvents to concentrate antioxidant compounds from methanolic extracts of evaluated plant materials. More detailed studies are required in order to identify the specific compounds responsible for this activity.

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## PHARMACEUTICAL TECHNOLGY

# ESTABLISHING SIMILARITY BETWEEN MULTISOURCE BETAHISTINE DIHYDROCHLORIDE ORAL DOSAGE FORMS USING *IN VITRO* METHODS

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**Abstract:** Betahistine dihydrochloride (BHD) is frequently used in treating attacks of vertigo in Ménière's syndrome. This study was conducted to classify BHD into biopharmaceutical classification system (BCS) and assess the suitability of using *in vitro* release profiles in demonstrating similarity between a generic and the innovator product. The solubility of BHD in aqueous media over the pH range of 1-7.5 was determined. Molecular descriptors like PSA, Log P, log D, and pKa were calculated using software packages. Tablets were tested for weight and content uniformity, friability and disintegration. The *in vitro* release profiles and dosage form performance of the generic were compared with the innovator. BHD was very highly soluble in aqueous media over the pH range of 1-7.5 and the dose number ranged from 0.0016 to 0.0048. The high permeability of BHD was predicted by molecular weight, number of hydrogen bond acceptors, number of hydrogen bond donors and the low PSA of 24.92 Å. Both, innovator and generic showed good dosage form performance and released more than 85% of their contents in less than 15 min. Our study demonstrated that BHD was a BCS class I drug and thus eligible for biowaiver applications. Similarity between immediate release oral dosage forms can be established using suggested *in vitro* dissolution methods.

**Keywords:** betahistine, dissolution, biopharmaceutical classification system, biowaiver

**Abbreviations:** API - active pharmaceutical ingredient, BCS - biopharmaceutical classification system, BE - bioequivalence, BHD - betahistine dihydrochloride, CHMP - Committee for Medicinal Products for Human Use, EMA - European Medicines Agency, FDA - United States Food and Drug Administration, IR - immediate release, Log D - distribution coefficient, Log P - n-octanol/water partition coefficient, MA - marketing authorization, PSA - polar surface area, RLD - reference listed drug, WHO - World Health Organization

Betahistine dihydrochloride (BHD) [N-methyl-2-(pyridin-2-yl)-ethanamine, 2HCl] (1) is a histamine-like (structural analogue) drug that is used clinically to decrease the severity and frequency of attacks of vertigo in the chronic or interim phase of Ménière's syndrome (2). Ménière's syndrome affects many people around the world. Recent studies reported a prevalence of 190 per 100,000 population in the Unites States alone (3, 4). Today, BHD is approved in more than 80 countries and since its first approval in 1968 more than 130 million patients used BHD (5). Pharmacodynamic studies have shown that betahistine acts as a neurotransmitter modulator of the complex histaminergic receptor system with strong antagonistic effect on the $H_o$ receptor (6, 7).

Various formulations are currently available on the market place around the world. Recently, there has been a growing concern on the quality and efficacy of generic formulations when clinicians and pharmacists are in difficult situations to choose among alternatives (8). The bioavailability of BHD from different oral formulations is an important parameter to compare the clinical performance of these formulations. Different formulations containing BHD might not have similar bioavailability and this may lead to suboptimal therapy when the formulation has less bioavailabil-

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ity or toxicity when the formulation has more bioavailability.

Since 1960s, *in vivo* pharmacokinetic bioequivalence (BE) studies have emerged as "gold standards" in proving similarity and interchangeability between innovator products and their generic versions. BE studies entail comparing the plasma or urine concentration *versus* time profiles of a test *versus* a reference listed drug (RLD) product (8). Sensitive methods to determine the concentration of BHD in biological samples after oral administration are still lacking (9). Previous methods reported in the literature were not sensitive enough to study the pharmacokinetics of BHD in plasma following oral administration (10). Since BHD undergoes almost compete first pass metabolism in human mainly to form 2-pyridylacetic acid (9), this metabolite can be used as a surrogate indication of BHD in human plasma following oral ingestion of BHD oral dosage forms (9). Recently, *in vitro* dissolution testing has emerged as powerful tool in predicting *in vivo* bioavailability of oral drug formulations. The advent of the biopharmaceutical classification system (BCS) and the wide adoption by the regulatory agencies around the globe, especially the United States Food and Drug Administration (FDA), European Medicines Agency (EMA) and the World Health Organization (WHO) have fundamentally changed the drug approval process for immediate release (IR) solid oral formulations (11). Regulatory agencies now can waive the *in vivo* BE studies for IR solid oral formulations containing high solubility (BCS class I and III) drugs and grant the formulation with a marketing authorization (MA) based on a biowaiver application (12, 13). Based on the biowaiver principles, very rapid dissolution or rapid dissolution with similarity factors are enough proof of similarity if the drug has a wide therapeutic index and the formulation contained non-interfering excipients (12-14).

The Focus Group on BCS and Biowaivers of the International Pharmaceutical Federation (FIP) has invited scientists around the world to prepare biowaiver monographs evaluating the suitability of waiving *in vivo* BE studies for drugs listed on the WHO's essential medicines list (15).

In the present study, we attempt to classify BHD as an active pharmaceutical ingredient (API) into its BCS solubility and permeability classes and we compare the *in vitro* dosage form performance of IR oral formulation containing BHD against the RLD. Our results provide rational for the interchangeability between the RLD and generic version based on *in vitro* release profiles. Our results might be of particular importance for pharmaceutical companies aiming to develop IR slid oral formulations containing BHD as an API, particularly concerned with the difficulty of quantifying pure BHD in biological fluids.

## EXPERIMENTAL

### Materials

Potassium dihydrogen orthophosphate, hydrochloric acid fuming (37%), and sodium hydroxide pellets GR for synthesis were purchased from Merck (Merck, Germany). BHD working standard was purchased from Refarmed (Refarmed Chemicals Ltd., Switzerland). Purified water was obtained using Millipore Milli-Q Plus (0.45 µm, conductivity: maximum 1.3 µs/cm, TOC: maximum 500 ppb) water purification system (Millipore, Bedford, USA). Generic BHD (24 mg) tablets were manufactured by a local pharmaceutical manufacturing company. The RLD for BHD (24 mg) tablets were purchased from a private pharmacy shop (Ramallah, Palestine).

### Determination of aqueous solubility

The solubility of BHD was determined using a standard shake flask method in the pH range of 1-7.5 at 37°C (16). Briefly, BHD was added in surplus amounts into aqueous media, the pH of the media was adjusted to 1, 3.5, 4.5, 6.8, and 7.5 using 0.01 M HCl or 0.01 M NaOH (13). The temperature was fixed at 37°C and flasks were shaken for 24 h. Samples were withdrawn and analyzed for BHD solubility using a double beam ultraviolet-visible spectrometer (PG Instruments, U.K.). The dose number was calculated according to the following equation (17, 18):

$$D_0 = \frac{(\frac{M_0}{V_0})}{C_s}$$

where, $M_0$ is the highest dose strength (milligrams), $C_0$ is the solubility (milligrams per milliliter), and $V_0$ = 250 mL.

### Determination of permeability

Molecular descriptors like polar surface area (PSA), *n*-octanol/water partition coefficient (log P), distribution-coefficient at pH 6 (log $D_o$), number of hydrogen bond acceptors, number of hydrogen bond donors and pKa of BHD were calculated using ACD/Labs (ACD/Labs, Advanced Chemistry Development: Toronto, Canada), ChemAxon (ChemAxon, Budapest, Hungary) and ALOGPS (The Virtual Computational Chemistry Laboratory, VCCLAB, Germany) software packages.

**Product characteristics and performance**

Product characteristics and performance were evaluated by standard weight uniformity, content uniformity, friability, disintegration and dissolution testing.

To evaluate weight uniformity, innovator and generic tablets were weighed individually using analytical balance (Ohaus, USA). The percentage deviation of the individual tablets from the mean was determined according to British Pharmacopeia (19). The friability strength of the uncoated tablets was estimated using a friability tester (Erweka TA3R, Germany). A double beam ultraviolet-visible spectrometer was used to quantify the amounts of BHD. Evaluation of disintegration time of BHD tablets was done according to the procedure described in the United States Pharmacopeia (20). Briefly, one tablet was placed in each of the 6 tubes of the basket of the disintegration apparatus (Erweka, Germany). Each tube was filled with water and the temperature was adjusted at 37 ± 2°C. Disintegration times were noted. According to the United States Pharmacopeia, IR tablets should disintegrate completely in ≤ 30 min.

Dissolution of RLD and generic BHD tablets was evaluated using United States Pharmacopeia paddle 2 semi-automated dissolution testing system (PT-DT70, PharmaTest, Germany). In accordance with the test standards recommended by the FDA, a total of 12 tablets were tested. In the dissolution apparatus, 1 tablet was placed in each vessel with a paddle stirrer at 50 rpm filled with 900 mL of dissolution media in three different pH points (0.1 M HCl, phosphate buffer pH = 4.5, and phosphate buffer pH = 6.8, respectively) and the temperature was adjusted at 37 ± 0.5°C (21). Aliquots of 10 mL from each dissolution vessel were removed after 10, 15, 20, 30 min) and substituted by the same volume. The amounts of BHD released were determined spectrophotometrically.

## RESULTS AND DISCUSSION

Interchangeability between a RLD and its generic versions is based on a proof of similarity (8). In this study, we attempted to classify BHD into BCS and assessed the similarity of a generic product containing BHD as an API with the RLD using *in vitro* release method. Pharmaceutical companies intending to formulate and market multisource IR solid oral dosage forms containing BHD might benefit from these results and apply for a waiver of *in vivo* BE studies (biowaiver) applications for the regulatory authorities, particularly when considering the difficulty of quantifying pure BHD in biological fluids.

**BCS class**

Our results showed that BHD was very highly soluble in water and the commercial dose of 24 mg can dissolve in less than 2 mL of water over the physiologically relevant pH range specified by the regulatory agencies (both FDA and EMA). According to the FDA guidelines, a drug can be considered highly soluble when the highest dose strength is soluble in 250 mL or less of aqueous media over the pH range of 1–7.5. This can be demonstrated through the calculation of the dose number of the drug of interest. Table 1 shows the solubility data and dose numbers at different pH points. Our results showed that BHD can unequivocally be assigned a “high solubility” BCS class. These solubility results were consistent with those reported in the literature (22-24). The estimated aqueous solubility of BHD was reported to be around 49 mg/mL (25). Similarly, in a previous application to waive *in vivo* BE requirements for BHD, the European Committee for Medicinal Products for Human Use (CHMP) evaluated the solubility data and concluded that BHD exhibit high solubility over the pH range specified in the EMA

Table 1. Solubility and dose number of BHD in aqueous media at 37°C over the pH range of 1-7.5.

| pH of the aqueous media | Solubility (mg/mL) | Dose number for 24 mg strength |
|---|---|---|
| 1 | 20 | 0.0048 |
| 3.5 | 30 | 0.0032 |
| 4.5 | 40 | 0.0024 |
| 6.8 | 60 | 0.0016 |
| 7.5 | 50 | 0.00192 |

guidelines (12). Our results showed that the dose number was less than 1 all over the pH range specified by the FDA too (1-7.5).

Table 2 shows calculated molecular descriptors of BHD using different software packages. Analyzing the chemical structure of BHD, ChemAxon predicted 2 hydrogen bond acceptors, 1 hydrogen bond donor, and a PSA of 24.92 Å. ChemAxon showed that BHD is a basic compound with a calculated pKa of 9.77.

The rest of molecular descriptors are shown in Table 2. Various attempts failed to quantify effectively pure BHD in the plasma (26). However, absorption was said to be rapid and complete following oral administration of radio-labeled betahistine with a peak plasma concentration reached 1 h after oral administration (9). The predicted PSA of BHD was less than 60 Å. Having a molecular weight of less than 500 (approximately 209), hydrogen bond acceptors of less than 10, and hydrogen bond donors of less than 5, BHD violates Lipinski's rule of five by only having a log *P* of less than 1 (27). Taken together, molecular weight, PSA with the number of hydrogen bond acceptors and hydrogen bond donors might explain the observed rapid and complete absorption of BHD *in vivo* (9, 27). Previous studies showed that PSA and log D best described intestinal oral absorption (28, 29). Although the reported log P values did not differ widely, it seems that partition coefficient data do not explain passive permeability, especially, when radio-labeled BHD showed rapid and complete absorption (9, 30-32). The extremely low concentrations of BHD in plasma following oral administration could be explained by the complete metabolism into 2-pyridylacetic acid (9, 30-32). BHD was shown to be excreted almost completely (80-90%) as 2-pyridylacetic acid in the urine within 24 to 48 h (26, 33). Taken together, our results suggest that BHD can be classified under BCS class I (high solubility and high permeability).

**Dosage form performance and *in vitro* release characteristics**

Visually inspected BHD tablets showed no defects in physical appearance. Overall, all tablets were of good quality and did not show any signs of defects with respect to shape, color, presence of black spots or preached edges. Previous studies showed that the organoleptic properties of a pharmaceutical product are very important for ensuring patient compliance and confidence in the therapy (34). As shown in Table 3, the tested tablets were within the accepted range for weight uniformity and disintegration time specified by the British Pharmacopeia (19). In this study, the content uniformity was also assessed to ascertain the quality of dosage form and ensure that

Table 2. Predicted molecular descriptors of BHD using different software.

| Property | Software | | |
|---|---|---|---|
| | ACD/Labs | ChemAxon | ALOGPS |
| Log P | 0.10 ± 0.21 | 0.63 | 0.59 |
| Log D at pH 6 (log $D_{6.0}$) | -2.99 | -2 | - |
| Number of hydrogen bond acceptors | 2 | 2 | - |
| Number of hydrogen bond donors | 1 | 1 | - |
| PSA (Å) | 24.92 | 24.92 | - |
| pKa | - | 7.4 | - |

Table 3. Characteristics of RLD and generic BHD tablets.

| Parameter | RLD | Generic |
|---|---|---|
| Weight average (mg) | 440 | 443 |
| Content assay (%) | 98.9 | 98.5 |
| Friability (% of loss) | < 1% | < 1% |
| Disintegration time (min) | 4.4 | 4.65 |

RLD = reference listed drug

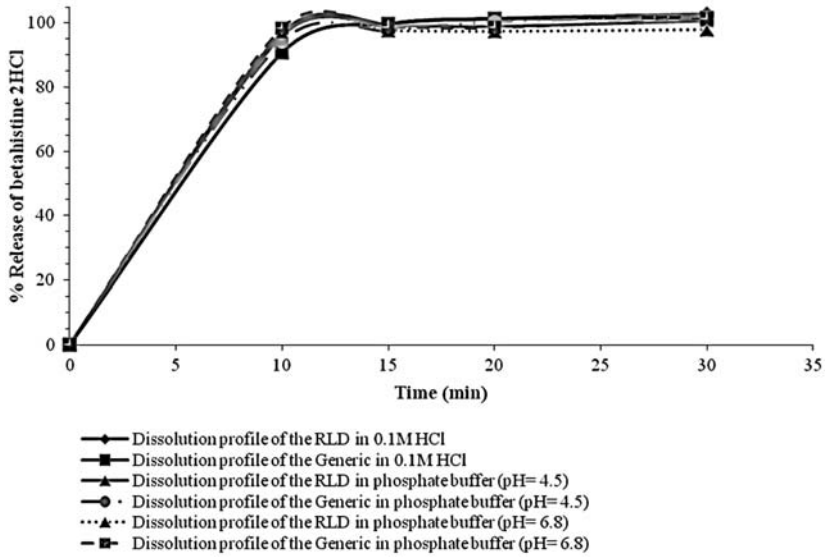


Figure 1. Dissolution profile of RLD and generic BHD in: 0.1 M HCl, phosphate buffer pH = 4.5 and phosphate buffer pH = 6.8

Table 4. Excipients included in multisource IR solid oral formulations with a MA in Canada (CA)[a], Czech Republic (CZ)[b], France (FR)[c], and United Kingdom (UK)[d], and the minimal and maximal amount of that excipient present per dosage unit in solid oral drug products with a MA in the US[e]

| Excipients | Drug formulations containing that excipient with a MA granted by the named country | Range present in solid oral dosage forms with a MA in the US (mg) |
|---|---|---|
| Povidone | CA (1), CZ (4-7, 9-12), FR (13-15, 17,19-23, 25), UK (26) | 0.17-80 |
| Microcrystalline cellulose | CA (1-3), CZ (4-12), FR (13-25), UK (26) | 0.75-1385 |
| Lactose monohydrate | CA (1), CZ (4-7, 9-12), FR (13-15, 17-25) | 4.9-614.2 |
| Colloidal anhydrous silica | CA (2), CZ (4-10), FR (13-25), UK (26) | 0.50-100 |
| Crospovidone | CA (1), CZ (4-7, 9-12), FR (13-15, 17-23, 25), UK (26) | 2-792 |
| Stearic acid | CA (1, 2), CZ (4-12), FR (13-25), UK (26) | 0.9–72 |
| Hydrogenated vegetable oil | CA (1, 11, 12) | 0.93-37.6 |
| Maize starch | CA (1, 11, 12) | 9.9–1135 |
| Mannitol | CA (2, 3, 8, 16, 18, 24), UK (26) | 10-454 |
| Talc | CA (2, 8, 16, 18, 24), UK (26) | 0.1–220 |
| Sodium stearyl fumarate | CA (2) | 0.85-29.3 |

[a]Source of data: www.hc-sc.gc.ca (accessed on 01-10-2014); [b]Source of data: www.sukl.cz (accessed on 01-10-2014); [c]Source of data: www.theriaque.org (accessed on 01-10-2014); [d]Source of data: www.mhra.gov.uk (accessed on 01-10-2014); [e]Source of data: FDA's Inactive Ingredient Database, http://www.fda.gov/Drugs/InformationOnDrugs/ucm113978.htm (version date: 29-03-2013). 1. Betahistine dihydrochloride tablets, 24 mg (Cobalt Pharmaceuticals); 2. Betahistine dihydrochloride tablets, 24 mg (Abbott Laboratories); 3. Betahistine dihydrochloride tablets, 24 mg (Teva Canada Limited); 4. Betahistine dihydrochloride tablets, 24 mg (Avefarm); 5. Betahistine dihydrochloride tablets, 24 mg (Actavis Group); 6. Betahistine dihydrochloride tablets, mg (Generics [UK] Limited, Potters Bar); 7. Betahistine dihydrochloride tablets, 24 mg (Ratiopharm GmbH); 8. Betahistine dihydrochloride tablets, 24 mg (Abbott Healthcare Products BV); 9. Betahistine dihydrochloride tablets, 24 mg (EGIS Pharmaceuticals PLC); 10. Betahistine dihydrochloride tablets, 24 mg (PharmaSwiss); 11. Betahistine dihydrochloride tablets, 24 mg (MEDOCHEMIE Ltd.); 12. Betahistine dihydrochloride tablets, 24 mg (HENNIG Arzneimittel GmbH & Co.); 13. Betahistine dihydrochloride tablets, 24 mg (ACTAVIS); 14. Betahistine dihydrochloride tablets, 24 mg (ARROW); 15. Betahistine dihydrochloride tablets, 24 mg (BIOGARAN); 16. Betahistine dihydrochloride tablets, 24 mg (BIPHAR); 17. Betahistine dihydrochloride tablets, 24 mg (EG); 18. Betahistine dihydrochloride tablets, 24 mg (MYLAN); 19. Betahistine dihydrochloride tablets, 24 mg (RANBAXY); 20. Betahistine dihydrochloride tablets, 24 mg (RATIOPHARM); 21. Betahistine dihydrochloride tablets, 24 mg (SANDOZ); 22. Betahistine dihydrochloride tablets, 24 mg (TEVA); 23. Betahistine dihydrochloride tablets, 24 mg (ZENTIVA); 24. Betahistine dihydrochloride tablets, 24 mg (BETASERC); 25. Betahistine dihydrochloride tablets, 24 mg (LECTIL); 26. Betahistine dihydrochloride tablets, 24 mg (Milpharm Limited).

Table 5. Summary of different bioequivalence studies of solid oral products containing BHD.

| Strength on which BE performed (mg) | Subjects | Test Formulation Manufacturer or License Holder | Composition | Reference product | Study design | Prandial | Bioequivalence Criteria/Statistics | Results | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| 24 1 | 7 | pms-Betahistine, Pharmascience Inc. | BHD 24 mg, crospovidone, colloida anhydrous silica,l lactose monohydrate, microcrystalline cellulose, povidone and stearic acid | Serc®, Solvay Pharma Inc. | A blind, randomized, two-way crossover study in healthy volunteers | Fasting | 90% CI ($C_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$)* | Bioequivalent | (27) |
| 24 | 24 | Teva-Betahistine, Teva Canada Limited. | BHD 24 mg, crospovidone, colloidal anhydrous silica, lactose monohydrate, microcrystalline cellulose, povidone and stearic acid | Serc®, Solvay Pharma Inc. | A blinded, single-dose, randomized, two-period, two-sequence, two-treatment, crossover study in healthy volunteers | Fasting | 90% CI ($C_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$)* | Bioequivalent | (38) |
| 24 | 36 | Vertisan, HENNIG Arzneimittel GmbH & Co. | BHD 24 mg, lactose monohydrate, corn starch, microcrystalline cellulose, citric acid, povidone, crospovidone type A, hydrogenated vegetable oil | Serc®, Solvay Pharma Inc. | A randomized, two-way crossover study in healthy volunteers | Fed | 90% CI ($C_{max}$, $AUC_{0-t}$, $AUC_{0-\infty}$)* | Bioequivalent | (38) |

*Since BHD undergoes almost compete first-pass metabolism in human, bioequivalence was established using parameters of the main metabolite, 2-pyridylacetic acid.

all tablets contain the same amounts of the API (BHD). Table 3 shows that the generic and RLD BHD tablets contain similar amounts in the range permitted by the British Pharmacopeia. Weight uniformity and content assay are important parameters ensuring the uniformity of dose for all patients and hence similar *in vivo* performance.

All tablet products were subjected to dissolution testing using dissolution media at pH of 1.2, 4.5 and 6.8. The dissolution profiles of the RLD and generic products are shown in Figure 1. All tested products showed very rapid dissolution and released ≥ 85% of their BHD contents in ≤ 15 min. Very rapid dissolving tablets are considered essentially similar without a need for similarity ($f_2$) and difference ($f_1$) factors (35).

**Excipients**

The excipients contained in several multisource IR solid oral formulations containing BHD 24 mg are listed in Table 4. The table does not include colorants, water, and ingredients present in the coating and polish. During our literature review, we did not find any evidence on possible effects of various excipients on the release and absorption of BHD. Since many of the multisource IR solid oral formulations containing BHD 24 mg are marketed in ICH associated countries, we assume that these formulations already passed rigorous BE studies and hence the combination of these various excipients do not affect the absorption of BHD. We therefore believe that there is no risk of bioinequivalence as a result of incorporating excipients or manufacturing process parameters.

***In vivo* BE**

Multisource BHD formulations were generally reported to perform well *in vivo*. In previous BE studies, plasma levels of the metabolite, 2-pyridylacetic acid, were compared in healthy volunteers and all formulations met the BE criteria compared to the RLD (33, 36, 37). Table 5 summarizes different BE studies conducted for some multisource IR solid oral formulations containing BHD as an active ingredient. Results showed that generics were bioequivalents compared to the RLD.

**Therapeutic index and patient's risk due to bioinequivalence**

Since its approval, clinical and postmarketing studies showed that BHD has a good safety profile (39). Safety surveillance data of more than 35 years revealed that the adverse drug reactions associated with the use of BHD are generally mild and serious reactions are very rare (39). The most commonly reported signs and symptoms were mild cutaneous hypersensitivity reactions (39). A few reported overdose cases (with up to 640 mg), showed that patients suffered mild to moderate symptoms of nausea, dry mouth, dyspepsia, abdominal pain and somnolence (27). Previous studies showed that even very high doses of 480 mg/day were beneficial for patients with severe Ménière's disease (40). The oral $LD_o$ for BHD was 3040 mg/kg in the albino rat (27). It is therefore unlikely that BHD would cause serious adverse reactions at concentrations moderately above or below the therapeutic concentrations as a result of bio*in*equivalence.

Furthermore, we searched two official lists of narrow therapeutic index drugs, one published by the National Institute of Health Sciences in Japan (http://www.nihs.go.jp) and the other by the United States FDA (http://ecapps.health.state.pa.us) and BHD was not found in these two lists. We therefore conclude that fluctuations in plasma levels and bioinequivalence might not present serious risk of toxicity since there is a wide difference between the usual therapeutic dose and toxic doses. So, it can be assumed that BHD is not a narrow therapeutic index drug and eligible for biowaiver applications.

## CONCLUSION

In conclusion, our investigation suggests that BHD is a high solubility, high permeability drug substance and hence assigned a BCS class I drug. BHD is not a narrow therapeutic index drug and the risks associated with bioinequivalence are manageable. Therefore, we can consider BHD as a suitable candidate for biowaiver applications and it is therefore suitable to use *in vitro* dissolution testing to surrogate *in vivo* BE testing. Establishing similarity between multisource IR oral formulations containing BHD as an API by comparing *in vitro* release profiles in dissolution media at the pH points of 1.2, 4.5 and 6.8 (at 37 ± 0.5°C) is scientifically justified when: 1) formulations contain only excipients that are well known and used in normal amounts known not to interfere with the release and absorption of the API, and (2) both the test and RLD formulation enable very rapid dissolution of BHD, or, rapid dissolution with similarity of the dissolution profiles demonstrated at least at pH 1.2, 4.5, and 6.8 for BHD.

**Competing interests**

All authors declare that this study was conducted for the purpose of scientific research only. All authors declare no competing interests.

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# THE RADIOLYTIC STUDIES OF CEFTRIAXONE IN THE SOLID STATE

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**Abstract:** The possibility of applying radiation sterilization to ceftriaxone disodium in the solid state was investigated. The lack of significant changes in the assay of ceftriaxone disodium irradiated with a dose of 25 kGy, required to attain sterility, was confirmed. The antibacterial activity of ceftriaxone disodium irradiated with a dose of 25 kGy, was unaltered for Gram-positive bacteria except of *Staphylococcus aureus* and changed for Gram-negative strains with except of *Klebsiella pneumoniae*. *Proteus vulgaris* showed the greatest sensitivity to CTD even after the application of radiation sterilization of 400 kGy.

**Keywords:** ceftriaxone disodium, radiation sterilization, stability

Ceftriaxone disodium (CTD) (Fig. 1) is a third-generation semisynthetic cephalosporin with a long half-life which has resulted in a recommended once daily administration schedule. It is administered intravenously or intramuscularly and has a broad spectrum of activity against Gram-positive and Gram-negative aerobic and some anaerobic bacteria. CTD is effective in complicated and uncomplicated urinary tract infections, lower respiratory tract infections, skin, soft tissue, bone and joint infections, septicemia and pediatric meningitis. In most of above mentioned infections once-daily administration appears efficacious.

CTD similarly to other cephalosporins have surprisingly few serious side effects, but the most of them are caused by the generation of degradation products. For example, fatal immune hemolytic anemia could be stimulated by a degradation product of ceftriaxone (1) so it is important to estimate the stability of cephems. The stability and mechanism of CTD degradation in aqueous solution (2) and in solid state (3) were investigated in previous studies. Other studies have confirmed that cephalosporins are susceptible to degradation in aqueous solutions (4-11) and in the solid state (3, 12-18). Our investigations focused on determining the influence of radiation on CTD at doses of 25 (recommended for sterility) (19), 50, 100, 200 and 400 kGy.

## EXPERIMENTAL

### Standards and reagents

Biotrakson is a sterile powder containing ceftriaxone disodium, for preparation of injections (Bioton Sp. z o.o. Warszawa, Poland). All other chemicals and solvents were obtained from Merck KGaA (Germany) and were of analytical grade. High quality pure water was prepared by using a Millipore Exil SA 67120 purification system (Millipore, Molsheim, France).

### Irradiation

Twenty-five mg samples of CTD were placed in 3 mL colorless glass vials that were closed with plastic stoppers. The samples in the vials were exposed to β irradiation in a linear electron accelerator LAE 13/9 (9.96 MeV electron beam and 6.2 μA current intensity) until they absorbed doses of 25, 50, 100, 200 and 400 kGy.

### Kinetic analysis

For the kinetic study, the Dionex Ultimate 3000 analytical system consisted of a quaternary pump, an autosampler, a column oven and a diode array detector. As the stationary phase a Kinetex with 5 μm core-shell particles, C18, 100A, 100 × 2.1 mm column was used. The mobile phase was com-

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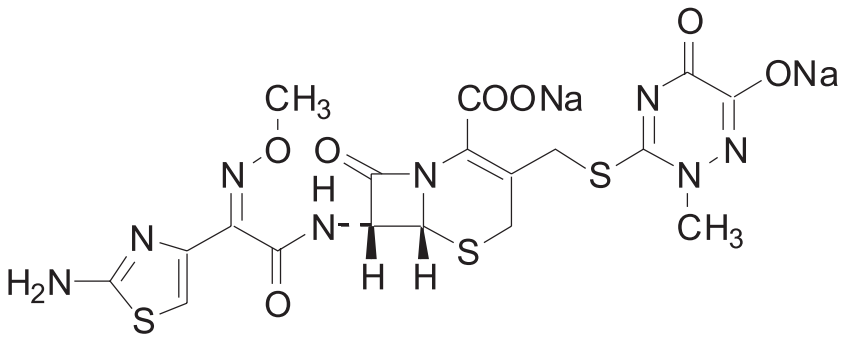

Figure 1. Chemical structure of ceftriaxone disodium

posed of acetonitrile – ammonium acetate (5 : 95, 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 254 nm. Separation was performed at 30°C.

The stability tests were performed according to the International Conference on Harmonization Guidelines (20).

**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 in aqueous solutions in conditions of acid, base and neutral hydrolysis and in the solid state (thermal and radiolytic degradation).

***Linearity***

Linearity was evaluated in the concentration range 42.4–508.8 mg/L (10-120% of the nominal concentration of CTD during degradation studies). The samples of each solution were injected three times and each series comprised 7 experimental points.

***Accuracy, as recovery test***

The accuracy of the method was determined by recovering CTD from the placebo. The recovery test was performed at three levels 80, 100 and 120% of the nominal concentration of CTD during degradation studies. Three samples were prepared for each recovery level. The solutions were analyzed and the percentages of recoveries were 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 CTD. Intermediate precision was studied comparing the assays performed on two different days.

***Limits of detection (LOD) and quantification (LOQ)***

The LOD and LOQ parameters were determined from the regression equation of CTD: 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.

***Robustness***

The robustness of the procedure was evaluated after changing the following parameters: the composition of the mobile phase; content of acetonitrile 5 ± 1 [%], the mobile phase flow rate 1.0 ± 0.2 [mL/min]; wavelength of detection 254 ± 5 [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 resulted in the essential changes of retention time and resolution in determination of CTD.

**Microbiological analysis**

Indicator microorganisms (*Salmonella enteritidis* ATCC 13076, *Salmonella typhimurium* ATCC 14028, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATTC 33420 and *Klebsiella pneumoniae* ATCC 31488) were cultured in soy-casein broth with yeast extract for microorganisms with increased nutritional requirements. *Clostridium butyricum* ATCC 860 and *Clostridium pasterianum* ATCC 6013 were grown in Reinforced Clostridial Medium (RCM, Oxoid, UK). Bacteria were cultured under aerobic or anaerobic conditions (37°C, 24 h) (depending on stains). Minimum inhibitory concentration (MIC) endpoints were determined by broth microdilution according to CLSI guidelines (21). The concentrations of irradiated CTD were 0.02–256 μg/mL. Then, irradiated CTD of decreasing concentrations were added to each of test tubes. Next, test tubes were inoculated with the same amount of cells suspension. After 16-18 h of incubation at 37°C, the growth of strains was checked *via* turbidity increase observation. In test tubes containing less than MIC of examined drugs the turbidity increase was observed (the cells have grown). The minimal concentration of drugs that inhibited strain growth was defined as MIC.

## RESULTS AND DISCUSSION

The HPLC method for determination of CTD was found selective in the presence of degradation product as shown in Figuress. 2 and 3. In the chro-

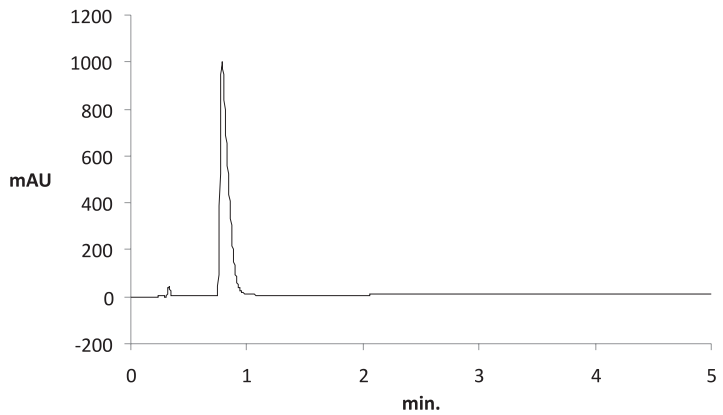


Figure 2. The HPLC chromatogram of irradiated CTD ($t_R$ = 0.787 min) at doses of 25 kGy (recommended for sterility)

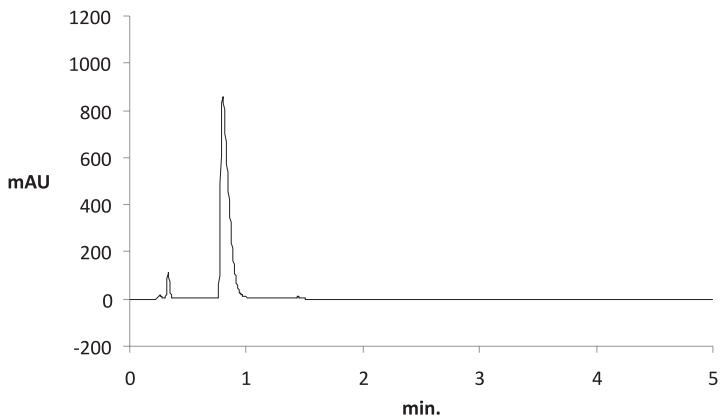


Figure 3. The HPLC chromatogram of irradiated CTD ($t_R$ = 0.787 min) at doses of 400 kGy

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

| Spiked concentration (mg/L) | Measured concentration ± S.D. (mg/L) | RSD (%) |
|---|---|---|
| Intra-day precision | | |
| 508.8 | 508.72 ± 0.02 | 0.0329 |
| 424.0 | 424.10 ± 0.06 | 0.0685 |
| 381.6 | 380.85 ± 0.04 | 0.0755 |
| Inter-day precision | | |
| 08.80 | 508.85 ± 0.07 | 0.0467 |
| 424.0 | 424.92 ± 0.08 | 0.0858 |
| 381.6 | 382.15 ± 0.06 | 0.1062 |
| Recovery studies | | |
| Spiked concentration (mg/L) | Measured concentration ± S.D. (mg/L) | Recovery (%) |
| 508.8 (~ 120%) | 509.11 ± 0.05 | 100.24 |
| 424.0 (~ 100%) | 423.95 ± 0.03 | 99.62 |
| 381.6 (~ 80%) | 381.59 ± 0.02 | 99.99 |

Table 2. Results of quantitative analysis of CTD before and after irradiation.

| Dose [kGy] | Content [%] |
|---|---|
| 0 | 100 |
| 25 | 97 |
| 50 | 94 |
| 100 | 93 |
| 200 | 91 |
| 400 | 80 |

matograms of CPS developed over a period of 0 to 5 min the following compounds were eluted: CTD with a retention time of 0.787 min and degradation product with retention time of 0.33 min. Peaks were symmetrical, clearly separated from each other (Figs. 2 and 3). The calibration plots were linear in the following concentration range 42.4–508.8 mg/L (n = 11, r = 0.9999). The calibration curve was described by the equation y = *a*x; y = (0.404 ± 0.002) x. The *b* value, calculated from equation y = *a*x + *b*, was not significant. Statistical analysis using Mandel's fitting test confirmed linearity of the calibration curves. The intra-day and inter-day precision values of measured concentration of CTD, as calculated from linearity plots are given in Table 1. The RSD values were from 0.0329 to 0.1062% demonstrating that the method was precise. The LOD and LOQ were 2.5 and 7.5 mg/L, respectively.

In contrast to the considerable instability of CTD under the influence of acid-basic hydrolysis (2), increased temperature and relative air humidity (3), after exposure to a radiation dose of 25 kGy CTD only 3% from initial concentration of CTD degraded (Table 2). A comparison of HPLC study results for non-irradiated and irradiated CTD samples demonstrated that the concentration of CTD changed after irradiation in higher doses (Table 2).

The effectiveness of radiation sterilization and possible changes in microbiological activity were also evaluated. The results of microbiological studies proved that irradiation at 25 kGy ensured the sterility of CTD samples. All tested species of reference strains showed sensitivity to CTD, both when non-sterilized and following different irradiation doses (Table 3). By comparing the activity of non-irradiated and irradiated CTD samples it was found that no significant differences between MIC values for the reference Gram-positive strains (*Listeria monocytogenes*, *Clostridium butyricum* and *Clostridium pasteurianum*) and Gram-negative *Klebsiella pneumonia* (Table 3) occur. On the other hand, CTD is active against *Listeria monocytogenes* and *Klebsiella pneumonia* only at high concentrations (Table 3). In the case of the reference strains (*Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*), the bactericidal properties of irradiated CTD samples were substantially reduced (Table 3). For *Staphylococcus aureus* even no inhibition of growth was observed. Among all the analyzed

Table 3. MIC values (ěg/mL) of irradiated CTD samples.

| Reference strain | CTD 0 kGy | CTD 25 kGy | CTD 400 kGy |
|---|---|---|---|
| Gram-negative bacteria | | | |
| *Salmonella enteritidis* ATCC 13076 | 0.24 | 32 | 32 |
| *Salmonella typhimurium* ATCC 14028 | 0.24 | 32 | 32 |
| *Escherichia coli* ATCC 25922 | 1 | 8 | 32 |
| *Klebsiella pneumoniae* ATCC 31488 | 32 | 32 | > 256 |
| *Pseudomonas aeruginosa* ATCC 27853 | 64 | 128 | 256 |
| *Proteus vulgaris* ATCC 33420 | 0.12 | 1 | 4 |
| Gram-positive bacteria | | | |
| *Clostridium butyricum* ATCC 860 | 4 | 4 | 16 |
| *Clostridium pasterianum* ATCC 6013 | 4 | 4 | 16 |
| *Staphylococcus aureus* ATCC 25923 | 8 | > 256 | > 256 |
| *Listeria monocytogenes* ATCC 7644 | 64 | 64 | 256 |

strains of the reference bacteria *Proteus vulgaris* showed the highest sensitivity to CTD, even in the variant, in which the irradiation dose applied on the investigated compound was 400 kGy. The difference connected with the sensitivity of Gram-negative (*Pseudomonas aeruginosa*) and Gram-positive bacteria (*Clostridium* sp.) could have resulted from the differences connected with cell wall structure, particularly since the bactericidal activity of CTD results from the inhibition of bacterial cell wall synthesis. Moreover, as a result of the effect of radiation sterilization, the spatial conformation of the compound could have changed and this may affect its activity towards the tested bacterial species. Taking into account differences between the sensitivities of HPLC and microbiological methods, the evaluation of CTD susceptibility to degradation was further studied at higher doses of 50, 100, 200 and 400 kGy. The CTD degradation rate increased with a radiation dose. Based on the HPLC analysis, a decrease in the CTD concentration connected with increasing radiation and the appearance of peaks originating from radiolysis products were observed (Figs. 2 and 3).

## CONCLUSIONS

The isocratic HPLC method developed for the analysis of CTD in pharmaceutical preparation 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. Microbiological methods, as they are more selective, should support HPLC methods for CTD quality control. The susceptibility to radiation of CTD limits the application of radiation sterilization as an alternative method to obtain sterile dosage forms of CTD. Changes in the concentration of CTD irradiated at 25 kGy cause a decrease in its microbiological activity against Gram-negative strains. Although that dose of radiation does not lead to any significant differences in the assay of CTD (3%), its bactericidal activity against *Salmonella enteritidis*, *Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Proteus vulgaris* was decreased.

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# INTERACTION OF WEAK BASE DRUG TRIMETAZIDINE AND CARBOPOL AS FURTHER RETARDATION IN THE MATRIX TABLET

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**Abstract:** Hydrophilic polymers using as matrix former in matrix tablets is a common approach and well-known excipient Carbopol is widely used for this reason too. In common case polymer doesn't interact with drug but Carbopol is a weak polyacrylic acid and obviously can interact at physiological enteric conditions with weak base drugs like trimetazidine dihydrochloride. During matrix tablet dissolution at enteric conditions, the microenvironment pH inside tablet changes from surface to center. It was found that hydrated matrix has unusual structured in microenvironment pH diapason of possible trimetazidine-Carbopol interactions. This matrix structuration resulted in significant matrix behavior changing and increasing trimetazidine release retardation in comparison with release data at gastric conditions. Thus, the example of trimetazidine-Carbopol interactions demonstrate additional mechanism of drug retardation that could be used for another appropriate weak base drugs.

**Keywords:** Carbopol, trimetazidine, matrix tablet, release retardation

In case of soluble matrices, a hydrogel formed after contact of matrix with medium and drug release occurs either *via* drug diffusion through a network of capillaries formed between compacted matrix former or/and erosion of the matrix. Dependent on the aqueous drug solubility, one of the mechanisms could dominate or combination of both takes place (1). Despite that Carbopol 71G is crosslinked polyacrylic acid and in principle is insoluble, the drug release occurs similarly to the water soluble matrices including erosion (2). Being a weak acid, Carbopol 71G can interact with weak bases at pH about pKa = 6.1. Trimetazidine dihydrochloride as a week base ($pKa_1$ 4.45, $pKa_2$ 9.14 (4)) can interact with Carbopol 71G. Therefore, the aim of this work was to investigate the trimetazidine-Carbopol interactions and their effect on drug release from matrix tablet.

## EXPERIMENTAL

### Materials

API: Trimetazidine dihydrochloride (TMZ × 2HCl, Sochinaz SA, Switzerland); matrix former: crosslinked polyacrylic acid (Carbopol 71G, Lubrizol Corp., USA); filler: lactose monohydrate (Granulac 200, Meggle AG, Germany); glidant: colloidal silicon dioxide (Aerosil 200 Ph, Evonik AG, Germany), lubricant: sodium stearyl fumarate (Pruv, JRS Pharma, Germany).

### Tablets preparation

Direct compression method was applied to obtain 200 mg biconvex tablets with 8 mm diameter according to the formulation presented in Table 1 using a mixer (Turbula T2F, Willy A. Bachofen AG, Switzerland) and eccentric tablet press (Korsch EKO, Korsch AG, Germany).

### Dissolution test

The drug release from tablets was investigated in a paddle apparatus (Vankel VK 300, Vankel Industries, Edison., NJ, USA) at following conditions: 900 mL of 0.1 M HCl or PBS pH 6.8, 100 rpm, 37°C; (n = 3). Samples were withdrawn at predetermined time points, filtered through 0.35 μm filters and measured UV-spectrophotometrically at λ = 269 nm (pH 1: $y = 0.0022x$, $R^2 = 0.9999$; pH 6.8: $y = 0.0022x + 0.0276$, $R^2 = 0.9993$).

### Aqueous solubility determination

The shake-flask method was used for TMZ × 2HCl and Granulac 200 solubility determination.

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Table 1. Tablet composition (% per 200 mg tablet)

| Formulation | F1 | F2 |
|---|---|---|
| TMZ × 2HCl | 17.5 | — |
| Granulac 200 | 31.3 | 48.8 |
| Carbopol 71G | 50 | |
| Aerosil 200 Ph. and Pruv | 0.2 and 1.0 | |

Table 2. Aqueous solubility of TMZ × 2HCl and Granulac 200 (n = 3, SD ≤ 5%)

| Compounds | Solubility (mg/mL) at pH corresponding to | |
|---|---|---|
| | Stomach | Small intestine |
| TMZ × 2HCl | 620 (pH 0.6) | 340 (pH 6.7) |
| Granulac 200 | 210 (pH 0.9) | 210 (pH 6.5) |

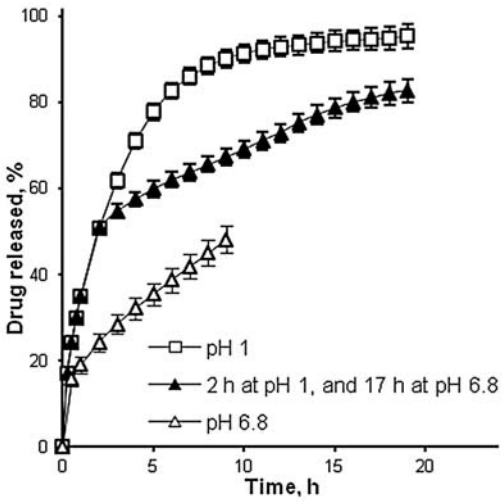



Figure 1. Effect of medium pH on drug release

The excess of tested substance was added to 50 mL of medium (0.1 M HCl or PBS pH 6.8). The equilibrium concentration was achieved in three days. The substance solubility was calculated after drying of known quantity of aliquot to constant weight at 105°C.

## RESULTS AND DISCUSSION

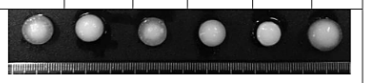
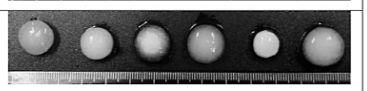
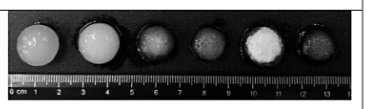
TMZ × 2HCl release from matrix tablets at pH 1 was much faster than at pH 6.8 (Fig. 1) or slowed down upon medium change from pH 1 to pH 6.8 after 2 h.

Since the solubility of Granulac 200 and TMZ × 2HCl is relatively pH independent in the range 1-6.8 (Tab. 2), the ionic interaction between positively charged TMZ and negatively charged Carbopol 71G could be a reason for slower drug release.

The swelling/erosion behavior of acidic dissolution medium (e.g., pH 1) of Carbopol 71G containing tablets was not affected by the presence of TMZ × 2HCl (Fig. 2). In this medium, Carbopol 71G was not ionized and no interaction with TMZ × 2HCl occurred. The release of freely soluble drug from swollen tablets was driven by diffusion and was relatively fast (Fig. 1).

In the medium with pH 6.8, approx. 80 % of carboxyl groups of Carbopol 71G and almost all tertiary amine groups of TMZ were ionized (according to $pKa_1$) and can interact with each other forming salt in a form of erodible gel layer (Fig. 3) on the surface of the tablet. Tablets containing TMZ (F1) did not swell in this medium in contrast to drug free (F2) tablets (Fig. 2).

The increased swelling and viscosity of ionized Carbopol 71G in the dissolution medium with pH 6.8 is well known phenomenon (3). However, due to interaction with ionized TMZ, drug containing tablets did not swell but rather eroded (Fig. 2). pH measurement of different regions of tablet cross-section after 5 h of dissolution test in phosphate buffer pH 6.8 showed a pH gradient inside of tablets (Fig. 3 D). Cone-shaped rolled strips of indicator paper which allows inserting the paper into a point was used to pH determination. The pH decreased from approx. 7 on the surface to 2-3 in the centre of the tablet. A thin erodible surface layer which contacts with PBS pH 6.8 organoleptically looks like mucus (Fig. 3 A). The pH 5-7 in outer layer corresponds to ionized state of Carbopol 71G and TMZ × 2HCl, where the interaction was possible. This outer layer has rubber-like structure with elastic properties (Fig.

| | pH 1 | | 2 h at pH 1 and 17 h at pH 6.8 | | pH 6.8 | |
|---|---|---|---|---|---|---|
| Time, h | F1 | F2 | F1 | F2 | F1 | F2 |
| 2 |  | | | | | |
| 5 |  | | | | | |
| 19 |  | | | | | |
| Dry residue* after 19 h, mg (n = 3; SD < 5%) | 112.0 | 100.8 | 57.8 | 28.1 | 107.2 | 22.4 |

* Dry residue was determined after drying to constant weight at 105°C

Figure 2. Matrix tablets behavior during dissolution test

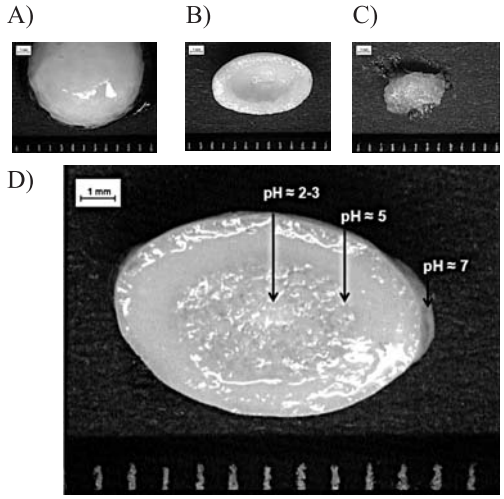



Figure 3. TMZ × 2HCl containing matrix tablet after 5 h of dissolution test at pH 6.8: A) whole tablet, B) separated rubber-like layer, C) separated gel core, D) cross-section

3 B). The central part of tablet cross-section, which corresponds to pH 2-5, has a form of plastic gel (Fig. 3 C) easy separable from outer layer. In contrast to TMZ tablets (F1), the drug free tablet (F2) in PBS pH 6.8 swelled without formation of internal structure and different way eroded (Fig. 3). After 19 h of dissolution test in PBS pH 6.8 (Fig. 2), the solid residue of drug free tablet (F2) was lower than solid residue of TMZ contain tablet (F1) in spite of lower solubility of Granulac 200 than TMZ × 2HCl.

The different behavior of TMZ containing tablet during dissolution test in 0.1 M HCl solution in contrast to PBS pH 6.8, the behavior difference of TMZ containing tablet in PBS pH 6.8 in contrast to drug free tablet, the internal structure formation of TMZ containing tablet in PBS pH 6.8 in contrast to drug free tablet allow us to ascertain the presence of TMZ-Carbopol 71G interaction. It seems that found interaction of Carbopol 71G and TMZ in the outer layer could be used for retardation of drug release.

## CONCLUSION

Slowdown of release in the release medium with pH 6.8 was due to the interaction of TMZ × 2HCl and Carbopol 71G with rubber-like layer formation. This interaction could be used for further retardation. Different release rate and mechanical properties of tablet in different physiological pH could provide problems for *in vitro*/*in vivo* correlation because of unpredictable tablet presence in the stomach. Therefore, one of the approaches to achieve this retardation in pH independent manner would be an enteric coating.

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# KINETICS AND MECHANISM OF DEGRADATION OF CEFOZOPRAN HYDROCHLORIDE IN THE SOLID STATE

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**Abstract:** The process of degradation was studied by using an HPLC-DAD method. Two degradation products were identified with a hybrid ESI-Q-TOF mass spectrometer. The influence of temperature and relative air humidity (RH) on the stability of cefozopran hydrochloride was investigated. In the solid state the degradation of cefozopran hydrochloride was a first-order reaction depending on the substrate concentration. The kinetic and thermodynamic parameters of degradation were calculated.

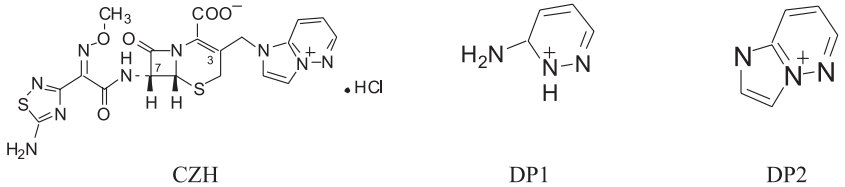
**Keywords:** cefozopran hydrochloride, HPLC, Q-TOF, stability, solid state

Cefozopran hydrochloride (CZH) (Fig. 1) is a parenteral, fourth-generation cephalosporin with broad spectrum activity against Gram-positive and Gram-negative organisms (1). Moreover, cefozopran has comparatively good activity against *Enterococci* and *Pseudomonas aeruginosa*, which are refractory to other cephalosporins (2). The main reason why CZH exhibits strong antimicrobial activity is assumed to be that it is stable against β-lactamase and that its action of inhibiting cell wall peptidoglycan cross-bridge formation is strong because it has a powerful affinity for penicillin binding proteins 1 and 2 of *Staphylococcus aureus* as well as for penicillin binding protein 3 of *Escherichia coli* and *P. aeruginosa* (3).

CZH has been clinically available for the treatment of various infections such as pneumonia, sepsis, urinary-tract and intra-abdominal infections in adult patients (2). CZH monotherapy is effective for the empirical treatment of pediatric cancer patients with febrile neutropenia (4-6). It is generally well-tolerated drug but fatal toxic epidermal necrolysis caused by CZH was observed (7). Probably, this adverse effect is caused by degradation products or impurities.

Chromatographic methods for the determination of CZH have proved the formation of many degradation products without their structural characterization (8-12).

The aim of this work was to investigate the kinetic of CZH degradation in the solid state and to identify degradation products.




CZH DP1 DP2

Figure 1. Chemical structures of CZH and its degradation products in solid state

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## EXPERIMENTAL

### Standards and reagents

CZH was obtained from CHEMOS GmbH, Regenstauf, Germany. It is a white or pale yellowish white, crystalline 98% pure powder soluble in water and conforms to the standards of Japanese Pharmacopoeia XV. All other chemicals and solvents were obtained from Merck KGaA (Germany) and were of analytical grade. High-quality pure water was prepared by using a Millipore Exil SA 67120 purification system (Millipore, Molsheim, France).

### Kinetic analysis

For the kinetic study, the 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 was composed of acetonitrile and 0.1% formic acid (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.

The stability tests were performed according to the International Conference on Harmonization Guidelines (13).

Five mg aliquots of CZH were weighed into glass vials. In order to test the influence of such environmental factors as temperature and humidity, the samples were placed in desiccators containing saturated solutions of inorganic salts: sodium bromide (RH ~50.9%), sodium nitrate (RH ~66.5%), sodium chloride (RH ~76.4%) and zinc sulfate (RH ~90.0%) that were in incubators (Wamed, Warszawa, Poland) set to the desired temperatures (333, 343, 353, and 363 K)

To evaluate the stability of CZH in dry air, the vials containing 5.0 mg of this substance were immersed in a sand bath placed in a heat chamber at 393 K.

Each batch to be studied comprised 8–12 samples. At specific time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and the contents dissolved in a mixture (1 : 1, v/v) of acetonitrile and water. The solutions obtained in that way were quantitatively transferred into volumetric flasks and diluted to a total volume of 10.0 mL with the same mixture of solvents. After filtration, the 10 µL samples were injected onto the column.

### LC-MS analysis

The LC-MS analysis was performed with the use of an Agilent Accurate-Mass Q-TOF LC/MS G6520B system with a DESI ion source and an Infinity 1290 ultra-high-pressure liquid chromatography system consisting of a G4220A binary pump, a G1330B FC/ALS thermostat, a G4226A autosampler, a G4212A DAD detector and a G1316C TCC module (Agilent Technologies, Santa Clara, USA). The chromatographic conditions were analogous to those described in the kinetic study. The MassHunter workstation software B.04.00 was used for the control of the system, data acquisition and qualitative analysis.

The Q-TOF detector was tuned in the positive (4 GHz) mode and the main parameters were optimized as follows: gas temp. 300°C, drying gas 10 L/min, nebulizer pressure 40 psig, capillary voltage 3500 V, fragmentor voltage 200 V, skimmer voltage 65 V, octopole 1 RF voltage 250 V. The data were acquired in the auto MS/MS mode with the mass range 50-1050 *m/z* and the acquisition rate 1.2 spectra/s (for MS and MS/MS data). The collision energy was calculated from the formula 2V (slope) × (*m/z*)/100 + 6 V (offset) and maximum 2 precursors per cycle were selected with an active exclusion mode after 1 spectrum for 0.2 min. To ensure accuracy in mass measurements, reference mass correction was used. Masses 121.0508 and 922.0097 were used as lock masses.

### Calculations

The rates constants of a first-order reaction were calculated from:

$$\ln c = \ln c_0 - k_{obs} \times t \qquad \text{(equation 1)}$$

where $c_0$ and c are concentrations at time t = 0 and t, respectively, and $k_{obs}$ is the observed rate constant of degradation (14).

Thermodynamic parameters ($E_a$, activation energy; $\Delta H^{\neq}$, enthalpy; $\Delta S^{\neq}$, entropy) were calculated from:

$$E_a = -a \times R \qquad \text{(equation 2)}$$

$$\Delta H^{\neq} = E_a - T \times R \qquad \text{(equation 3)}$$

$$\Delta S^{\neq} = R \times (\ln A - \ln(k_B \times T/h)) \qquad \text{(equation 4)}$$

where: $k_B$ = Boltzmann's constant ($1.3807 \times 10^{-23}$ J × $K^{-1}$); h = Planck's constant ($6.626 \times 10^{-34}$ J × s); R = universal gas constant (8.314 $K^{-1} \times mol^{-1}$), T = temperature [K]; a = slope of the Arrhenius relationship; A = frequency coefficient where: (ln A = b) (14).

Statistical parameters of the respective equations were calculated using Microsoft Excel 2010.

## RESULTS AND DISCUSSION

Changes in the concentration of CZH under stress study conditions were evaluated using the

Table 1. Q-TOF accurate mass elemental composition and MS/MS fragmentation of the analyzed substances.

| Comp. No. | Name | Retention time (min) | Measured mass (*m/z*) | Theoretical mass (*m/z*) | Mass error (ppm) | Molecular formula $[M+H^+]$ | MS/MS fragmentation (*m/z*) | Fragmentation ions formulas |
|---|---|---|---|---|---|---|---|---|
| 1 | DP1 | 1.22 | 96.05512 | 96.05562 | 5.26 | $C_4H_6N_3$ | 81.95996<br>68.05020<br>55.95761 | $C_4H_6N_2$<br>$C_4H_6N$<br>$C_3H_3N$ |
| 2 | CZH | 4.1 | 516.08805 | 516.08668 | 2.65 | $C_{19}H_{18}N_9O_5S_2$ | 369.04325<br>325.05264<br>167.02687<br>120.05525 | $C_{12}H_{13}N_6O_4S_2$<br>$C_{11}H_{13}N_6O_2S_2$<br>$C_7H_7N_2OS$<br>$C_6H_6N_3$ |
| 3 | DP2 | 5.3 | 120.05543 | 120.05562 | 1.61 | $C_6H_6N_3$ | 93.04294<br>79.02758 | $C_5H_5N_2$<br>$C_4H_3N_2$ |

HPLC method and were linear in the range 20-300 mg/L, accurate (RSD 99.78-100.85 %), precise (RSD 0.12-0.47 %) and selective in the presence of CZH and its degradation products. The LOD and LOQ values were 6.72 and 20.51 mg/L, respectively. In the chromatograms of CZH developed over a period of 0 to 10 min, the following compounds were eluted: CZH with a retention time ($t_R$) of 4.1 min, degradation products (DP1) with $t_R$ = 1.22 min and DP2 with $t_R$ = 5.3 min.

The degradation of CZH in the solid state was a first-order reaction depending on the substrate concentration. The rate constants were determined from obtained results by using the least squares method for the equation:

$$\ln c_t = \ln c_0 - k_{obs} \times t$$

where $c_t$ and $c_0$ are the concentrations of CZH (y) at time t = 0 and t (x), respectively, and $k_{obs}$ is the rate constant of the degradation reaction. The following statistical parameters of the equations were calculated by using the least squares method: $y = ax + b$, $a \pm \Delta a$, $b \pm \Delta b$, standard deviations $s_a$, $s_b$, $s_y$ and the coefficient of the linear correlation r ($y = c_t$, x = time). The values of error range of slope ($\Delta a$) and intercept ($\Delta b$) were obtained for f = n – 2 degrees of freedom, with $\alpha = 0.05$.

Other 4th generation cephalosporin: cefpirome sulfate (CPS) and cefepime dihydrochloride monohydrate (CPH) are degraded with the same kinetic mechanism as CZH (15, 16). Ceftriaxone disodium (CTD) and cefoselis sulfate (CSS), despite structural similarities to CZH, are degraded according to an autocatalytic reaction of the first order with respect to substrate concentration at increased RH and temperature (17, 18).

High-resolution LC-MS analysis allowed identification of the parent compound as well as degradation products of CZH in the solid state. As shown in Table 1, the molecular ions of CZH and its degradation products were found with very high accuracy (1.61–5.26 ppm), which permitted calculation of the chemical formulas for all the analyzed compounds. Additionally, the MS/MS fragmentation spectra that were obtained (main fragmentation ions are listed in Table 1) confirmed the proposed structures of all of the identified substances (Fig. 1).

Based on the kinetic mechanism of degradation identified as a first-order reaction depending on the substrate concentration, it may be assumed that products of CZH degradation (Fig. 1) do not have any catalytic effect on the degradation process in the solid phase.

The effect of RH on the stability of CZH at 363 K is presented in Table 2. As expected, RH turned

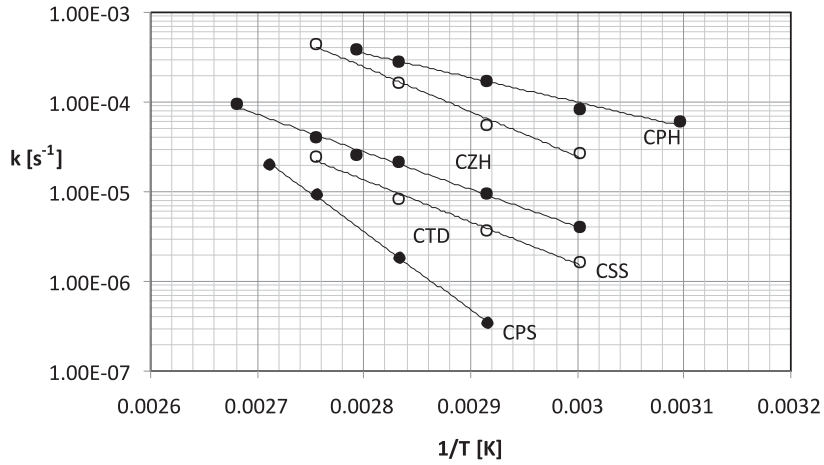


Figure 2. Semilogarithmic relationship k = f(1/T) for degradation of CZH, CPS (14), CPH (15), CTD (16) and CSS (17) at RH ~ 76.4%; CZH, CPS, CPH and CSS (4th generation cephalosporins), CTD (3rd generation cephalosporin)

Table 2.The effect of relative air humidity on the stability of CZH at 363 K.

| Relative air humidity (%) | $10^{-1}$ (k ± Δk) [$s^{-1}$] | Statistical evaluation ln k = f(RH%) |
|---|---|---|
| 50.9 | (0.71 ± 0.16) | $a$ = 0.06 ± 0.03 |
| 66.5 | (2.33 ± 0.24) | $S_a$ = 6.95 × 10 |
| 76.4 | (4.52 ± 0.21) | $b$ = -12.50 ± 2.16 $S_b$ = 0.50 |
| 90.0 | (7.46 ± 0.60) | r = 0.9871 $S_y$ = 0.20 |

Table 3. Kinetic and thermodynamic parameters of degradation of CZH in solid state at RH ~ 76.4%.

| T [K] | $10^5$ (k ± Δk) [$s^{-1}$] | Statistical evaluation ln k = f(1/T) | Thermodynamic parameters |
|---|---|---|---|
| 333 | (2.72 ± 0.19) | $a$ = -11472.35 ± 3621.09 | $E_a$ = 95.385 ± 30.107 [kJ $mol^{-1}$] |
| 343 | (5.60 ± 0.53) | $S_a$ = 841.52 | $\Delta H^{\neq a}$ = 92.907 ± 30.107 [kJ $mol^{-1}$] |
| 353 | (16.08 ± 0.84) | $b$ = 23.81 ± 10.42 | $\Delta S^{\neq a}$ = – 46.93 ± 86.65 [$JK^{-1}$ $mol^{-1}$] |
| 363 | (45.18 ± 2.09) | $S_b$ = 2.42<br>r = 0.9947 $S_y$ = 0.15 | |

[a] calculated for 298 K.

out to be key factor determining the degradation of CZH. The more rapid degradation of CZH at increased RH in comparison to CPS, CSS and CTD (Fig. 2, Table 4) resulted from the susceptibility of the C-3 and C-7 substituents to degradation under the influence of humidity. Therefore, the structure of those substituents is responsible for the pharmacological properties and stability of CZH in the solid state. In this study, the impact of temperature on CZH stability at increased RH was also investigated. The values of the reaction rate constants $k_{obs}$ were used to calculate the Arrhenius relationship in order to interpret the influence of the temperature on the reaction rate at 76.4% RH (Fig. 2). The energy of activation and the thermodynamic parameters, enthalpy and entropy for 298 K, were calculated from the parameters of the slope ln $k_i$ = f(1/T) (Table 3). In the solid state at RH ~ 76.4% CPS had the highest activation energy ($E_a$) and CPH the lowest. Nevertheless, CSS was the most stable of all the compared cephalosporins, most likely due to a different kinetic mechanism of degradation. The stabil-

Table 4. Kinetic and thermodynamic parameters of degradation of CZH, CPS (15), CPH (16), CTD (17) and CSS (18) in solid state at constant relative air humidity (RH ~ 76.4%) and at T= 363 K

| Cephem | $E_a$, [kJ mol$^{-1}$] | Half-life ($t_{0.5}$) [h] | Kinetic mechanism of degradation |
|---|---|---|---|
| CZH | 95 ± 30 | 0.4 | first-order reaction depending on the substrate concentration |
| CPS | 167 ± 16 (15) | 20.2 (15) | first-order reaction depending on the substrate concentration (15) |
| CPH | 52 ± 12 (16) | 0.4* | first-order reaction depending on the substrate concentration (16) |
| CTD | 79 ± 9 (17) | 23.1 (17) | autocatalytic reaction of the first order with respect to substrate concentration (17) |
| CSS | 90 ± 26 (18) | 34.5 (18) | autocatalytic reaction of the first order with respect to substrate concentration (18) |

*calculated from extrapolated data

ity of CZH was very similar to that of CPH and was the lowest of the compared cephems.

## CONCLUSIONS

The degradation of CZH in the solid state in dry air and at increased relative air humidity is a first-order reaction. The kinetic mechanism of CZH degradation does not depend on storage conditions. The degradation products of CZH do not have catalytic effect on the degradation process in the solid phase.

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**PHARMACOLOGY**

# CITALOPRAM AND VENLAFAXINE DIFFERENTIALLY AUGMENTS ANTIMICROBIAL PROPERTIES OF ANTIBIOTICS

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**Abstract:** We investigated intrinsic antibacterial potential of citalopram (CIT) and venlafaxine (VF) alone and in combination with seven antibiotics against clinical isolates of Gram positive and Gram negative bacteria, ATCC strains and to evaluate their effect on reversal of antibiotic resistance. Intrinsic antibacterial action of CIT, VF and MICs were determined using well assay, nutrient broth and agar dilution techniques. Disk diffusion method was used to study bacterial susceptibility to CIT and VF. Intrinsic antibacterial assay revealed that CIT possesses mild to moderate intrinsic bactericidal properties, while VF was found inactive. CIT and VF augmented the antibacterial effects of antibiotics and some previously resistant strains were converted to susceptible range. Antibacterial activities of levofloxacin, moxifloxacin and gentamicin were significantly increased against *S. aureus* in combination with 310 µg/mL of CIT. Moxifloxacin, levofloxacin and gentamicin activity against *E. coli* was significantly increased with the addition of 600 and 1200 µg/mL of VF, respectively ($p < 0.05$). Resistance of *E. coli* to cefixime and *P. aeruginosa* to cloxacillin were reversed with addition of increasing concentration of CIT. Further, resistance of *S. aureus* ATCC 6538 to cefixime, *E. coli* to cefixime, cloxacillin and *P. aeruginosa* resistance to cloxacillin were reversed with increasing concentrations of VF. CIT and VF also showed activity against clinical isolates of *Salmonella typhi, E. coli, Klebsiella pneumoniae, Proteus mirabilis, S. aureus, P. aeruginosa* and *Enterococcus faecalis*, exhibiting minimum inhibitory concentrations from 6 to 7 mg/mL. Combination study revealed that these drugs are resistance modifying agents in combination with antibiotics.

**Keywords:** intrinsic antibacterial assay, ATCC, MDR, citalopram, venlafaxine

Antimicrobial drug resistance is a major challenge in the chemotherapy of infectious diseases and cancer (1, 2). Factors leading to multidrug resistance (MDR) include, reckless utilization, sustained over reliance on antimicrobial agents, target site modification and active drug efflux mediated by efflux pumps (3, 4). To overcome MDR, antibiotics are administered along with inhibitor drugs, which will restore the activity of antibiotics by inhibition of inactivating enzymes (5) or increase the availability of antibiotics at the target site by reducing their extrusion out of the target site (6, 7).

Different compounds used for the management of non-infectious pathological conditions known to exhibit antimicrobial activity against a variety of microbes are known non-antibiotics (8). Among the non-antibiotic compounds, several have been reported to synergistically interact and augment the activity of antibiotics against a variety of microorganisms (8, 10). In the recent years, it is reported that selective serotonin reuptake inhibitors (SSRIs) demonstrate antibacterial characteristics (11) and can reverse *Plasmodium* resistance to antimalarial drugs (12). Selective serotonin reuptake inhibitors are

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mainly effective against Gram positive microbes, yet are less effective against the majority of Gram negative bacteria (11). Sertraline - an SSRI, has been reported to possess antifungal activity against *Candida* species (13). The same group of investigators reported that a group of SSRI (fluoxetine, sertraline, paroxetine, citalopram) and nor-epinephrine reuptake inhibitor (NRI) reboxetine, inhibit the growth of *Aspergillus* species *in vitro* (14).

Phenothiazines and antidepressants have been reported to reverse multidrug resistant phenotypes of bacteria and make them susceptible to previously resistant antibiotics (15, 17). Paroxetine and femoxetine are known to potentiate antimicrobial activity of several antibiotics against Gram positive bacteria (18). Kaatz et al. investigated the activity of these compounds and their derivatives against *S. aureus* and *E. coli* (19). The assayed compounds augmented the effect of various antimicrobial agents against multidrug resistance efflux pumps including NorA, non NorA, AcrAB-TolC pumps and inhibited the activity of RND efflux pumps (20). Paroxetine in a concentration of 25 µg/mL afforded eight fold decrease in minimum inhibitory concentration (MIC) of norfloxacin against *S. aureus* 1199B a NorA producing strain and k2068 a non-NorA phenotype (19). Antimicrobial activities of three phenothiazines: promazine, thioridazine, triflupromazine and two tricyclic antidepressants: amitriptyline and imipramine were investigated by Hendricks et al., against *S. aureus*, *P. aeruginosa* and *Klebsiella pneumonia* (21). The phenothiazine derivatives thioridazine and triflupromazine showed notable activity against *S. aureus* and *P. aeruginosa* strains. Evidence also indicates that SSRIs inhibit the expression of P-glycoprotein's, primary active transporters responsible for the extrusion of several chemotherapeutic agents from their site of action (22). Based on these investigations, some newly developed antidepressants including CIT and VF were selected for further antibacterial evaluation alone and in combination with antibiotics.

## EXPERIMENTAL

### Chemicals and drugs

Citalopram (CIT) and venlafaxine (VF) were kindly provided by Universal Pharmaceuticals and Usawa Pharmaceuticals, Pakistan, respectively. Antibiotic powder of ciprofloxacin, levofloxacin, norfloxacin, moxifloxacin, cefixime, cloxacillin, and gentamicin were purchased from Sigma Aldrich, USA. Antibiotic discs (Oxoid) and dimethyl sulfoxide (DMSO) Labscan Patumwan, Bankok 10330, Thialand, were used in the study.

### Bacterial strains

Control strains including *S. aureus* (ATCC 6538), *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 9027) were kindly provided by CIRIN Pharmaceuticals (Pvt.) Ltd., Pakistan. Clinical isolates were collected from Microbiology Department of Khyber Teaching Hospital (KTH) Peshawar, Pakistan and were identified by different biochemical tests (23). Bacteria were preserved in freeze-dried condition at 4$^{o}$C in stab slant agar until later use.

### Culture media

Nutrient agar (Oxoid Ltd., UK), mannitol salt agar (Oxoid Ltd., England, CM0085), MacKonkeys agar (Oxoid Ltd., England, CM0115), triple sugar iron (TSI) agar (Oxoid Ltd., England, CM0277), CLED medium (Oxoid Ltd., England, CM0423), DNASE agar (Oxoid Ltd., England, CM0321), Simmons citrate agar (Oxoid Ltd., England, CM0155), urea agar base (Oxoid Ltd., England, CM0053), nutrient broth base powder (Oxoid) were used for the growth and identification of the selected microorganisms according to the guidelines of Clinical Laboratory Standards Institute (CLSI) and manufacturer specifications.

### Preparation of stock solutions

Depending on solubility, CIT was dissolved in dimethyl sulfoxide (DMSO) and serial twofold dilutions were made, ranging from 310 to 5000 µg/mL under laminar flow hood. VF solutions were prepared in distilled water ranging from 75 to 1200 µg/mL. Sterilization of the stock solutions were performed by passing it through syringe filters of Minisart (Sartorious) 0.2 µm size in safety cabinets.

### Standardization of bacterial suspension

Bacterial cultures were grown for 24 h at 37$^{o}$C and suspensions with cell density of $10^{8}$ CFU/mL, were prepared using McFarland standard and further diluted to a cell density of $10^{6}$ CFU/mL using a UV visible spectrophotometer (Thermo Electron Corporation, USA) at 625 nm and the standardization was maintained for the period of the study.

### Intrinsic antibacterial studies on citalopram and venlafaxine

Intrinsic antibacterial action of CIT and VF were determined using agar dilution and well assay methods. In agar dilution method, CIT and VF solutions corresponding to 310-5000 µg/mL and 75-1200 µg/mL, respectively, were aseptically added to sterile molten MHA at 40$^{o}$C (24). One loopful of the

already prepared bacterial suspension was inoculated on the MHA plates containing increasing concentration of CIT and VF. Plates were incubated at 37°C for 24 h and were examined for the appearance of growth. In well assay method, bacterial plates were inoculated by swabbing MHA plates with already prepared bacterial suspensions under laminar flow hood (25, 27). Wells of 6 mm diameter were bored into the MHA plates using sterilized cork borer. After drying the bores were filled with 100 µL of CIT, VF and antibiotics solutions taking care not to let spillage of the solutions on the surface of the agar. The plates were incubated at 37°C for 24 h. Zone of inhibition were measured around the bores.

**Determination of minimum inhibitory concentrations (MICs)**

For determination of MICs both nutrient broth and agar dilution methods approved by CLSI were used (28, 29). For these tests, CIT in concentration of 310-5000 µg/mL and VF 75-1200 µg/mL were added to sterilized tube containing nutrient broth and were inoculated with the test microbes. Tubes were incubated using shaker incubator at 37°C for 24 h. MIC was considered that concentration at which no visible bacterial growth was observed. All experiments were done in five replicates.

***In vitro* synergy between citalopram, venlafaxine and antibiotics**

The combined antibacterial effect of CIT, VF and antibiotics were determined by disk diffusion method as described by CLSI (30). Sterile filter paper disks (Whatman no. 1, 7.25 mm diameter) were prepared using method described previously (28). In combination study, solutions corresponding to 5 µg of ciprofloxacin, levofloxacin, moxifloxacin, cefexime, cloxacillin, 10 µg of norfloxacin and gentamicin, 310-500 µg of CIT and 75-1200 µg of VF were added to these disks. Antibiotic disks of these drugs available were used as positive control.

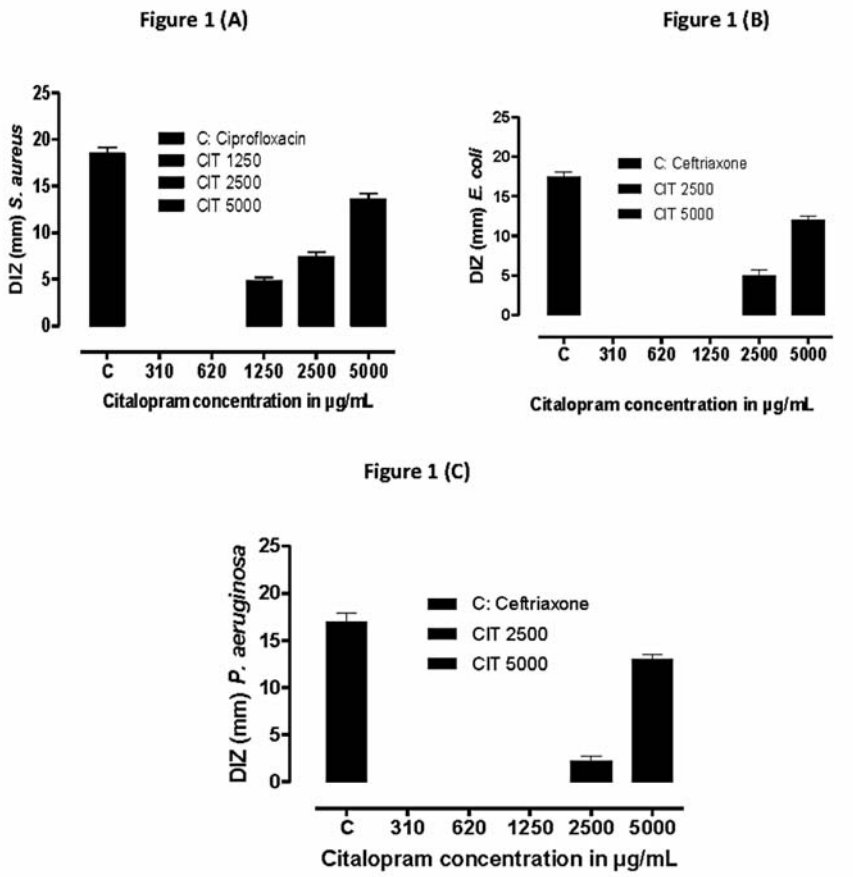


Figure 1. Intrinsic antibacterial activities of citalopram against *Staphylococcus aureus* (A), *Escherichia coli* (B) and *Pseudomonas aeruginosa* (C)

Table 1. Diameter of inhibitory zones of selected antibiotics with increasing concentrations of citalopram (310-5000 µg/mL) against *S. aureus* ATCC 6538.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + CIT 310 µg/mL | Antibiotic + CIT 620 µg/mL | Antibiotic + CIT 1250 µg/mL | Antibiotic + CIT 2500 µg/mL | Antibiotic + CIT 5000 µg/mL |
| Ciprofloxacin 5 µg | 27.50 ± 0.57 | 29 ± 0.81 | 30.75 ± 0.50* | 34.25 ± 0.95*** | 39 ± 0.00*** | 43 ± 2.30*** |
| Levofloxacin 5 µg | 24.25 ± 0.50 | 27.5 ± 1.00*** | 30 ± 0.0*** | 32.5 ± 0.57*** | 37.5 ± 0.57*** | 40 ± 0.00*** |
| Norfloxacin 10 µg | 24.25 ± 0.50 | 25.75 ± 0.50 | 28.5± 0.57*** | 31.25 ± 0.5*** | 37.5 ± 1.73*** | 40 ± 0.00*** |
| Moxifloxacin 5 µg | 29 ± 0.00 | 31.5 ± 0.57** | 34.5 ± 1.0*** | 36.5 ± 1.29*** | 38.5 ± 0.57*** | 40.5 ± 0.57*** |
| Cefixime 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Cloxacillin 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Gentamycin 10 µg | 28.75 ± 0.95 | 30.50 ± 0.57** | 35 ± 0.00*** | 39.25 ± 0.95*** | 40.5 ± 0.57*** | 42.5 ± 0.57*** |

*: Values significantly different as compared to antibiotic treatment group only, *p < 0.05, ** p < 0.01, *** p < 0.001. Control: antibiotic only. CIT: citalopram.

Table 2. Diameter of inhibitory zones of selected antibiotics with increasing concentrations of venlafaxine (VF 75-1200 µg/mL) against *S. aureus* ATCC 6538.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + VF 75 µg/mL | Antibiotic + VF 150 g/mL | Antibiotic + VF 300 µg/mL | Antibiotic + VF 600 µg/mL | Antibiotic + VF 1200 g/mL |
| Ciprofloxacin 5 µg | 28.6 ± 3.13 | 32.6 ± 3.97 | 33.6 ± 3.97 | 34 ± 3.74 | 35.8 ± 3.19* | 39.8 ± 1.09*** |
| Levofloxacin 5 µg | 27.8 ± 1.09 | 32.2 ± 3.27 | 33.8 ± 3.19 | 34 ± 3.39* | 35.6 ± 3.36** | 38.8 ± 1.64*** |
| Norfloxacin10 µg | 25.4 ± 0.89 | 29.8 ± 3.19 | 32 ± 3.93* | 34 ± 3.46** | 34.6 ± 3.97** | 40.2 ± 4.14*** |
| Moxifloxacin 5 µg | 28.6 ± 2.07 | 33.6 ± 2.07** | 34.4 ± 1.94** | 35.4 ± 1.140*** | 37.2 ± 2.16*** | 39.6 ± 3.20*** |
| Cefixime 5 µg | 0 ± 0 | 5.2 ± 4.816 | 5.4 ± 5.07 | 7 ± 4.12* | 10.4 ± 0.54*** | 12.6 ± 1.34*** |
| Cloxacillin 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Gentamycin 10 µg | 29.6 ± 0.54 | 33 ± 1.22 | 34.6 ± 1.67* | 35.2 ± 1.30*** | 35.4 ± 1.34*** | 38.2 ± 3.42*** |

*: Values significantly different as compared to antibiotic treatment group only, *p < 0.05, ** p < 0.01, *** p < 0.001. Control: antibiotic only. VF: venlafaxine.

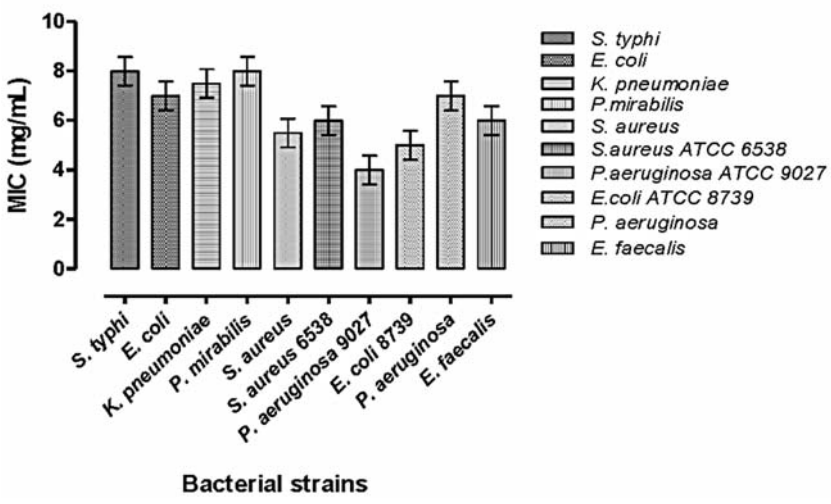


Figure 2. Minimum inhibitory concentrations (MICs) of citalopram against bacterial strains

Overnight grown bacterial culture was used to prepare bacterial suspensions. From these suspensions 100 µL were uniformly spread over the surface of already prepared MHA plates under laminar flow hood and were allowed to dry. Disks containing antibiotic alone and increasing concentrations of CIT and VF were placed equidistantly on the surface of inoculated plated and were incubated at 37°C for 24 h. Diameter of inhibitory zones of antibiotic alone and in combination with CIT and VF were determined according to CLSI standards for zone interpretation (31).

**Statistical analysis**

One-way ANOVA followed by Dunnett's multiple comparison test were applied for the comparison of positive control with the test groups. All the experiments were repeated in five replicates and values were expressed as the means ± S.E.M. The p values less than 0.05 were considered as statistically significant.

## RESULTS AND DISCUSSION

**Intrinsic antibacterial activity and MICs of citalopram and venlafaxine**

In intrinsic antibacterial study, CIT exhibited moderate activity, whereas VF was completely devoid of intrinsic activity even at highest concentration used (Fig. 1 A,B). Intrinsic activity of CIT was more prominent against Gram-positive bacteria i.e., *S. aureus* (Fig. 1A, 2) and inhibitory zones were increased along the concentration of CIT scoring inhibitory zone of 5, 8 and 14 mm at concentrations of 1250, 2500 and 5000 µg/mL, respectively. CIT at concentrations of 2500 and 5000 µg/mL produced inhibitory zones of 5 and 12 mm, respectively, against *E. coli* while against *P. aeruginosa* CIT was only effective at higher concentration (5000 µg/mL) scoring inhibitory zone of 13 mm. MICs of CIT against of *S. aureus* ATCC 6538, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, were 4, 5, and 4 mg/mL, respectively, whereas MICs for clinical isolates were: *Salmonella typhi* 6 mg/mL, *E. coli* 7 mg/mL, *K. pneumonia* 6 mg/mL, *P. mirabilis* 6 mg/mL, *S. aureus* 5 mg/mL, *P. aeruginosa* 6 mg/mL and 7 mg/mL for *Enterococcus faecalis* 29212 (Fig. 2). MICs of VF against these bacteria were too high to be clinically important and were above concentrations that were used in combination with antibiotics.

**Effect of increasing concentrations of citalopram and venlafaxine on the susceptibility of *S. aureus***

The activity of antibiotics were significantly improved with the addition of increasing concentrations of CIT and VF (Tables 1, 2). The combined anti-staphylococcal activity was more pronounced for levofloxacin, moxifloxacin and gentamicin, whereby only 310 µg/mL of CIT has significantly increased the diameter of inhibitory zones $p \leq 0.001$, $\leq 0.01$ and $\leq 0.01$, respectively, in comparison to antibiotic treated groups only. *S. aureus* was found to be resistant to cefixime and cloxacillin and this resistance was not reversed even at the highest concentration being used. VF was hypothesized to be

Table 3. Diameter of inhibitory zones for selected antibiotics with increasing concentrations of citalopram (CIT 310-5000 µg/mL) against *E. coli* ATCC 8739.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + CIT 310 µg/mL | Antibiotic + CIT 620 µg/mL | Antibiotic + CIT 1250 µg/mL | Antibiotic + CIT 2500 µg/mL | Antibiotic + CIT 5000 µg/mL |
| Ciprofloxacin 5 µg | 26.0 ± 1.41 | 26.5 ± 0.70 | 28.5 ± 0.70 | 30.0 ± 1.41* | 30.5 ± 0.70* | 36.5 ± 2.12*** |
| Levofloxacin 5 µg | 25.5 ± 0.70 | 26.5 ± 0.70 | 27.5 ± 0.70 | 29.5 ± 0.70** | 31.0 ± 1.41** | 34.5 ± 0.70*** |
| Norfloxacin 10 µg | 24.0 ± 0.00 | 24.0 ± 0.00 | 25.5 ± 0.70 | 27.5 ± 0.70** | 30.0 ± 0.00*** | 32.0 ± 0.70*** |
| Moxifloxacin 5µg | 26.5 ± 0.70 | 26.5 ± 0.70 | 28.0 ± 0.00 | 29.5 ± 0.70* | 30.5 ± 0.70** | 34.0 ± 0.70*** |
| Cefixime 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 10.0 ± 0.00 |
| Cloxacillin 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Gentamycin 10 µg | 23.0 ± 2.82 | 26.0 ± 1.41 | 28.0 ± 1.41* | 30.0 ± 0.00* | 31.5 ± 0.70** | 35.5 ± 0.70*** |

*: Values significantly different as compared to antibiotic treatment group only, *p < 0.05, ** p < 0.01, *** p < 0.001. Control: antibiotic only. CIT: citalopram.

Table 4. Diameter of inhibitory zones of selected antibiotics with increasing concentrations of venlafaxine (VF 75-1200 µg/mL) against *E. coli* ATCC 8739.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + VF 75 µg/mL | Antibiotic + VF 150 g/mL | Antibiotic + VF 300 µg/mL | Antibiotic + VF 600 µg/mL | Antibiotic + VF 1200 g/mL |
| Ciprofloxacin 5 µg | 26.8 ± 6.26 | 28.2 ± 7.15 | 30.6 ± 6.50 | 32.2 ± 5.11 | 34.4 ± 5.54 | 36.2 ± 2.94 |
| Levofloxacin 5 µg | 25.4 ± 5.81 | 27 ± 5.83 | 28 ± 6.44 | 29.2 ± 4.38 | 32.2 ± 3.89 | 34.4 ± 2.88* |
| Norfloxacin 10 µg | 22.6 ± 8.41 | 24 ± 8.63 | 25.6 ± 8.56 | 26.4 ± 7.02 | 29.6 ± 5.54 | 33 ± 3.00 |
| Moxifloxacin 5 µg | 29.4 ± 2.79 | 31.2 ± 2.77 | 31.8 ± 2.48 | 32.8 ± 2.58 | 34 ± 2.00* | 35.8 ± 1.48** |
| Cefixime 5 µg | 12.8 ± 11.51 | 16.8 ± 11.51 | 18.2 ± 2.55 | 19.6 ± 12.83 | 22.2 ± 8.87 | 21.80 ± 8.34 |
| Cloxacillin 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 4.8 ± 1.024 | 7 ± 1.47 | 10 ± 2.47 |
| Gentamycin 10 µg | 25.8 ± 3.03 | 27.4 ± 3.84 | 29 ± 4.35 | 29.8 ± 4.60 | 30.6 ± 4.56 | 33.4 ± 4.66* |

***:** Values significantly different as compared to antibiotic treatment group only, * p < 0.05, ** p < 0.01. Control: antibiotic only. CIT: citalopram.

better antibacterial as its has dual reuptake inhibitory mechanism (SNRI) in humans and could have inhibitory effect on efflux pumps. Being devoid of intrinsic antibacterial activity at concentrations range used (75-1200 µg/mL), it has significantly increased the activities of all antibiotics except cloxacillin in concentration dependent manner. *S. aureus* ATCC 6538 was resistant to cefixime alone but in the presence of VF its susceptibility was increased scoring an inhibitory zone of 12 mm (Table 2).

Resistance of *S. aureus* to various antibiotics develops due to the presence of NorA and other efflux pumps (32). Kaatz et al. reported that selective serotonin reuptake inhibitors has the ability to block this pump and reverse antibiotic resistance (33). The increase in susceptibility of *S. aureus* to the selected antibiotics may be due to physical interaction of CIT with NorA efflux pump and may be equally contributed to intrinsic antimicrobial activities of CIT. Also it was observed that there was attenuation of microbial growth in CIT added plates indicating its direct inhibitory effects on *S. aureus*. Our laboratory is still working to find out mechanism of CIT and VF induced bacterial death and their possible role as efflux pump inhibitors.

**Effect of increasing concentrations of citalopram and venlafaxine on the susceptibility of *Escherichia coli***

Antibacterial activity of CIT and VF are summarized in Tables 3, 4. The combined antibacterial activity of CIT was more marked against Gram-positive bacteria (*S. aureus*) in comparison to Gram-negative (*E. coli and P. aeruginosa*). CIT at a concentration of 1250 µg/mL has significantly increased the antibacterial activity of selected drugs except cloxacillin and cefixime. *E. coli* initially resistant to cefixime become susceptible at 5000 µg/mL of CIT scoring an inhibitory zone of 10 mm. Though, this inhibitory effect was not statistically significant but might have an uncovered reversal mechanism involved there. The increase in antibacterial activity of VF was moderate against *E. coli*. Although diameter of inhibitory zones increased steadily along the concentration gradient but significant increase was observed only for levofloxacin, moxifloxacin and gentamicin at higher concentration. *E. coli* susceptibility to cloxacillin and cefixime was increased with the addition of VF to whom it was previously resistant. Among the two drugs, CIT was more effective in combination with antibiotics against *E. coli* as compared to VF. Researchers usually favor those compounds which are intrinsically inert but can synergize the antibacterial effect of other drugs. Thus, further studies on VF and derivation are necessary to uncover possible antibiotic-VF combinations and how to minimize neurological effects at effective concentrations.

**Effect of increasing concentrations of citalopram and venlafaxine on the susceptibility of *Pseudomonas aeruginosa***

The vulnerability of *P. aeruginosa* to ciprofloxacin and norfloxacin was significantly increased with the addition of only 310 µg/mL of CIT, whereas levofloxacin and moxifloxacin exhibited synergistic interactions with CIT at 620 µg/mL (Tables 5 and 6). *P. aeruginosa* was resistant to cloxacillin initially, but with the addition of increasing concentrations of CIT its susceptibility was increased indicated by an increase in the diameter of inhibitory zone. Significant synergy between gentamicin and CIT was observed at 2500 µg/mL and resistance to cefixime was not affected at any concentration of CIT being used. VF at concentration of 600 µg/mL has significantly increased the inhibitory zones of ciprofloxacin, levofloxacin, norfloxacin and moxifloxacin against *P. aeruginosa*. Interestingly, its susceptibility to cloxacillin and cefixime has increased with the addition of VF. The exact mechanism of the antibacterial activity of these drugs is still not known, however it is hypothesized that as these compounds are pump inhibitors (SSRI & SNRI) in humans so they may inhibit the activity of bacterial efflux pumps. These compounds may also work by competing with the substrates thus reducing the function of these pumps. A more recent approach is that these inhibitor compounds have affinity for the substrates of MDR pumps. So, by forming an appropriate complex of antibiotic with these inhibitors can inhibit its efflux due to competitive inhibition or larger size.

## CONCLUSIONS

The widespread multidrug resistance among pathogens necessitates the development of resistance modifying agents, which when combined with antimicrobial drugs will augment the activity of these agents and will prevent the emergence of acquired resistance. Drugs in combination (i.e., amoxicillin and clavulanic acid) will act synergistically to augment the effectiveness of drug therapy and give broad spectrum of activity. So it is required to interact antibiotics with non-antibiotic compounds to find out synergistic or potentiating characteristics of these drug combinations. These com-

Table 5. Diameter of inhibitory zones in mm of selected antibiotics with increasing concentrations of citalopram (310-5000 µg/mL) against *P. aeruginosa* ATCC 9027.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + CIT 310 µg/mL | Antibiotic + CIT 620 µg/mL | Antibiotic + CIT 1250 µg/mL | Antibiotic + CIT 2500 µg/mL | Antibiotic + CIT 5000 µg/mL |
| Ciprofloxacin 5 µg | 25.2 ± 0.95 | 28 ± 1.41* | 29 ± 1.41** | 30.2 ± 0.95*** | 31.5 ± 1.00*** | 33.2 ± 1.50*** |
| Levofloxacin 5 µg | 24.5 ± 0.57 | 26.5 ± 1.00 | 28.5 ± 2.081** | 30 ± 1.41*** | 31.2 ± 1.25*** | 34.5 ± 0.57*** |
| Norfloxacin 10 µg | 22.5 ± 1.00 | 25.7 ± 1.50* | 27.5 ± 1.73*** | 28.5 ± 1.73*** | 30 ± 1.15*** | 31.7 ± 1.50*** |
| Moxifloxacin 5 µg | 25.7 ± 0.95 | 27.5 ± 1.29 | 29.5 ± 1.29** | 30.5 ± 1.29*** | 30.5 ± 1.29*** | 32 ± 1.41*** |
| Cefixime 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 |
| Cloxacillin 5 µg | 0 ± 0.00 | 0 ± 0.00 | 0 ± 0.00 | 4.5 ± 1.00 | 7 ± 1.42 | 10.2 ± 1.50 |
| Gentamycin 10 µg | 24 ± 0.81 | 25.2 ± 0.95 | 27 ± 1.82 | 28 ± 1.82 | 28.7 ± 2.06* | 30.2 ± 3.59** |

*: Values significantly different as compared to antibiotic treatment group only, *p < 0.05, ** p < 0.01, *** p < 0.001. Control: antibiotic only. CIT: citalopram.

Table 6. Diameter of inhibitory zones of selected antibiotics with increasing concentrations of venlafaxine (VF 75-1200 µg/mL) against *P. aeruginosa* ATCC 9027.

| Antibiotic | Diameter of the inhibitory zone (mm); the mean ± SEM (n = 5) | | | | | |
|---|---|---|---|---|---|---|
| | Control | Antibiotic + VF 75 µg/mL | Antibiotic + VF 150 g/mL | Antibiotic + VF 300 µg/mL | Antibiotic + VF 600 µg/mL | Antibiotic + VF 1200 g/mL |
| Ciprofloxacin 5 µg | 27.25 ± 2.36 | 29 ± 2.58 | 30.2 ± 2.98 | 30.7 ± 2.87 | 33 ± 2.44* | 35 ± 2.16** |
| Levofloxacin 5 µg | 26.75 ±1.50 | 28.2 ± 0.95 | 28.7 ± 1.25 | 29.5 ± 1.91 | 31.5 ± 1.91** | 33.7 ± 1.25*** |
| Norfloxacin 10 µg | 24 ±3.55 | 27 ± 4.24 | 27.5 ± 3.31 | 29 ± 3.82 | 31.7 ± 2.87* | 33.5 ± 1.73** |
| Moxifloxacin 5 µg | 27.25 ±0.50 | 30.2 ± 2.87 | 31.5 ± 3.31 | 32 ± 2.82 | 33.5 ± 2.38* | 34.7 ± 2.50** |
| Cefixime 5 µg | 0 ± 0.00 | 4.5 ± 1.00 | 7.2 ± 1.50 | 8.5 ± 0.50 | 10.7 ± 1.50 | 14 ± 1.50 |
| Cloxacillin 5 µg | 5 ± 0.70 | 6 ± 0.97 | 7.7 ± 0.67 | 9.2 ± 0.58 | 10.5 ± 0.79 | 11 ± 1.61 |
| Gentamycin 10 µg | 25.50 ± 3.31 | 29 ± 7.74 | 30.5 ± 8.18 | 32.2 ± 8.50 | 34.5 ± 7.93 | 37 ± 6.63 |

*: Values significantly different as compared to antibiotic treatment group only (Control), *p < 0.05, ** p < 0.01, *** p < 0.001. VF: venlafaxine.

binations will ultimately lead to broad spectrum drugs thus reducing cost and duration of antimicrobial drug therapy. The main reason behind this study was long term use of these antidepressant drugs and antimicrobial history of antidepressants and antipsychotics. From antibacterial studies of CIT and VF it is concluded that these possess antibacterial properties and both increased the activity of antibiotics in concentration dependent manner. VF being inactive intrinsically has potentiated antibacterial activities of antibiotics in combination while CIT has exhibited synergistic interactions with antibiotics. Resistance to some antibiotics is also reversed with the addition of these drugs. The daily recommended dose of CIT is 20-30 mg and can be increased up to 60 mg, whereas daily recommended dose of VF is 75-375 mg daily (34). As this is first screening of CIT and VF for possible antibacterial potentials, so high concentrations were also tested to find out useful range in relation to antimicrobial activity. However, from bioavailability point of view, any relevance between our *in vitro* results and *in vivo* performance is still not clear and may require further *in vivo* studies to investigate the mechanism of CIT and VF induced bacterial death. These antidepressants were very effective at higher concentration but achieving such concentrations clinically and neurological aspects will be a challenge. Further derivatization and use of bacteria containing molecularly characterized efflux pumps can provide more convincing results. Studies regarding structural information and possible efflux pumps inhibitory potential of these compounds in the presence and absence of known efflux pump inhibitors is in progress in our laboratory.

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**Competing interests**

The authors declare that they have no competing interest.

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# CHANGES OF SALIVARY CORTISOL LEVEL AFTER VENLAFAXINE TREATMENT

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**Abstract:** Depression is one of the widespread diseases. Antidepressants used in the therapy of depression reduce the cortisol level but the impact of venlafaxine on the hormone secretion has not been explained so far. For this reason, the purpose of this study was to learn to what extent the venlafaxine therapy can affect the salivary cortisol level. Day-by-day cortisol quantitation in saliva of twenty depressed women was performed by HPLC. Statistical analysis and pattern recognition approach were used for interpretation of the results. Venlafaxine in mono- and combined therapy with sertraline, trazodone or mianserin has been found to have an impact on cortisol secretion as shown by the mean initial and final salivary cortisol levels, which were 76.01 ± 83.35 and 8.22 ± 7.15 ng/mL, respectively. The highest and lowest cortisol levels also indicate that fluctuation of the hormone secretion decreases during hospitalization and there are some different profiles of cortisol secretion as shown by the cluster and principal component analyses. The results have shown that venlafaxine used in combined therapy strongly reduced fluctuation of the cortisol level. For instance, for patients in which the fluctuation was found the mean hospitalization period was 42 days, whereas those treated with venlafaxine in monotherapy were hospitalized for almost 48 days. In conclusion, the combined therapy distinctly reduced the time of healing.

**Keywords:** cortisol, depressed women, pattern recognition approach, saliva, venlafaxine

Depression, one of the most common diseases around the world, can be caused by many factors. One of the theories claims depression as being due to long-lasting stress associated with increasing level of glucocorticosteroids (1). This is the effect of dysregulation of the hypothalamic pituitary adrenal (HPA) axis. Glucocorticosteroids have a profound effect on hippocampal cell proliferation, which is one of two brain regions where robust neurogenesis continues into adulthood. Another factor which has a crucial impact on the neurogenesis is stress, which reduces it. The nerve cells in hippocampal formation are the most sensitive to the deleterious effects of stress owing to enhanced release of glucocorticoids.

It has been found in the literature that in both depression and stress, an increase in cortisol secretion occurs (1). A crucial impact on the cortisol secretion have antidepressants, including selective serotonin reuptake inhibitors (SSRI) and serotonin and norepinephrine reuptake inhibitors (SNRI). Among SNRI, venlafaxine (VEN) is a commonly used drug in the therapy of depression. The mechanism of VEN action depends on the dosage used. A study of the impact of VEN on HPA axis by its serotoninergic effect and on cortisol secretion revealed that the hormone level in the healthy men blood increases proportionally to the dosage (2). The rise in cortisol secretion was also observed in saliva of healthy men and women, but at the beginning of night only (3). In the morning, its level fell down to the reference value. Furthermore, the low doses of VEN act as selective serotonin reuptake inhibitors and similarly to these drugs affect the cortisol secretion in healthy volunteers (4). The study has also shown that after 6 weeks of VEN therapy, in the majority of patients the cortisol level was lowered and the health was improved. At the same time, in the late responders group an insignificant reduction of the depression symptoms was observed, and additionally, the secretion of adrenocorticotropin hormone was still enhanced.

The differences were also observed in the impact of VEN therapy on cortisol secretion between responders and non-remitting patients (5).

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The results of cortisol quantification in saliva before therapy and repeatedly during the therapy showed that the hormone level was not lowered both in the depressed patients and the control group of healthy subjects. No significant impact of VEN on salivary cortisol was also shown by Hallam et al. (3). On the other hand, a significantly lower afternoon salivary cortisol was observed in the remitting than in non-remitting patients. Another theory on the increased level of cortisol during the depression hinges on variations in the activity of intracellular modulators of steroid activity, especially of two intracellular cortisol-deactivating enzymes. During the therapy with VEN, the levels of both enzymes returned to the level of control group (6).

Because VEN is a commonly used drug in the therapy of depression, it is interesting to monitor its influence on the changes of cortisol level during hospitalization of patients with major depressive disorder. This is crucial owing to the necessity of lowering the cortisol level during depression treatment. For this reason, the aim of this study was to learn to what extent the VEN therapy can affect variations in the salivary cortisol level. This purpose is justified by the fact that only a sparse information on the impact of VEN therapy on the cortisol secretion is available in the literature. In addition, these data are often controversial.

For quantitation of the cortisol level in the depressed patients a saliva has been chosen as diagnostic material (7, 8) to eliminate the influence of blood sampling stress on the hormone level. According to the literature data, a strong correlation between variations of the cortisol concentration in blood and saliva has been found to be of crucial significance for this study (9–11).

## EXPERIMENTAL

### Participants

The subjects with major depressive disorder (MDD) were recruited at the Hospital for Nervous and Mental Diseases in Starogard Gdański (Poland). All the participants were examined by a psychiatrist using the clinical interview and diagnosed according to the International Classification of Diseases (ICD-10) (12) criteria. All the subjects were informed in detail about the purpose of the study and gave their written consent to participate in it. The subjects were informed that they could discontinue the course at any time, if desired.

Some of the participants with MDD were excluded if their medical condition precluded administration of venlafaxine or if they had serious health problems, such as adrenal function disorders. Some other subjects were excluded because of the pregnancy and breastfeeding and if their participation in the course could be detrimental to their well-being. The participants were also excluded if they were unable to understand the nature of the study after discussion with a research nurse.

Finally, 20 women with MDD, 15 treated with VEN in monotherapy (MT) and 5 in combination therapy (CT) were included into the study. Table 1 summarizes the demographic and clinical characterization of the subjects, and a detailed information on the antidepressants and the doses used. The study had been approved by the ethical committee of the Medical University of Gdansk (Poland).

### Quantitation of salivary cortisol

Salivary samples for quantitation of cortisol were collected from depressed women day-by-day during the whole hospitalization period, including the first day. The samples were collected into plastic tubes without any stimulation at 10 a.m. and frozen. The subjects were instructed to rinse their mouths with water, not to eat or drink 30 min before the samples have been collected.

For quantitation of salivary cortisol, a procedure of isolation and analysis has been developed by Dziurkowska and Wesolowski (13). The efficiency of cortisol extraction from saliva indicated that the recovery exceeded 94%. Validation of the HPLC method has shown that the coefficients of variation for the intra-day and inter-day studies varied from 1.1 to 6.5% and did not exceed 6.8%, respectively. The recovery fell in the range of 93.6 to 100.8%.

### Statistical methods

All calculations were performed using a Statistica 7.1 (StatSoft, Kraków, Poland) software. The Wilcoxon test was used to study the impact of VEN therapy on salivary cortisol level in depressed women. With the aid of this test, the initial and final cortisol concentrations as well as the mean levels of the hormone during three periods of hospitalization, were compared. The influence of antidepressant therapy on the time of hospitalization was also checked. A one-way analysis of variance (ANOVA) test was used to study the correlation between the VEN dose and hospitalization period. To control the effect of VEN dosage on the mean concentration of salivary cortisol during three periods of hospitalization, ANOVA test for repeated measurements was used. The level of statistical significance was set at $p < 0.05$.

Two unsupervised pattern-recognition methods, cluster (CA) and principal component (PCA)

Table 1. Characteristics of the patients and the influence of venlafaxine in monotherapy and combined therapy on the cortisol level in saliva.

| No. | Age of subjects (year) | Multiplicity of hospitalization | Dose used (mg) | Hospitalization period (days) | Cortisol concentration in saliva (ng/mL) | | Highest and lowest cortisol concentrations in successive periods of hospitalization | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | initial | final | 0–30% | 31–60% | 61–90% |
| 1 | 51 | 4 | 75 | 90 | 173.62 | 0.25 | 0.25–173.62 | 0.25–40.00 | 0.25–18.75 |
| 2 | 52 | 1 | 75 | 28 | 158.75 | 11.25 | 2.50–158.75 | 2.50–22.50 | 2.75–31.25 |
| 3 | 47 | 2 | 75 | 31 | 41.25 | 8.75 | 1.62–50.00 | 3.75–36.25 | 2.50–32.50 |
| 4 | 57 | 1 | 75 | 63 | 15.00 | 9.87 | 1.25–32.50 | 1.25–62.50 | 1.75–13.50 |
| 5 | 47 | 2 | CT | 28 | 32.00 | 3.12 | 2.75–32.00 | 3.75–18.75 | 2.50–20.12 |
| 6 | 36 | 1 | 75 | 26 | 24.75 | 9.00 | 17.12–24.75 | 1.25–13.12 | 9.00–10.00 |
| 7 | 29 | 1 | 75 | 34 | 372.50 | 11.87 | 32.00–372.50 | 7.50–17.50 | 4.75–11.87 |
| 8 | 53 | 2 | 75 | 40 | 91.50 | 20.00 | 5.50–91.50 | 2.50–36.25 | 3.12–20.00 |
| 9 | 53 | 1 | 150 | 50 | 75.00 | 5.25 | 3.87–75.00 | 2.50–32.50 | 2.62–57.50 |
| 10 | 44 | 2 | CT | 46 | 41.00 | 3.62 | 2.50–41.00 | 1.50–10.00 | 1.25–23.75 |
| 11 | 44 | 2 | 75 | 87 | 40.00 | 9.25 | 1.75–40.00 | 2.50–30.62 | 2.62–32.50 |
| 12 | 47 | 3 | CT | 39 | 5.62 | 1.50 | 4.75–12.12 | 2.75–12.12 | 1.50–10.12 |
| 13 | 59 | 1 | CT | 52 | 95.00 | 11.37 | 4.87–95.00 | 4.75–32.50 | 2.50–11.37 |
| 14 | 26 | 1 | 75 | 68 | 90.00 | 7.25 | 3.25–90.00 | 2.87–15.00 | 3.25–11.50 |
| 15 | 47 | 1 | 150 | 14 | 70.00 | 3.25 | 27.50–70.00 | 6.00–58.75 | 3.25–6.00 |
| 16 | 48 | 2 | 75 | 18 | 84.75 | 3.50 | 5.62–84.75 | 4.25–6.50 | 3.50–7.50 |
| 17 | 52 | 1 | 75 | 36 | 15.00 | 2.62 | 1.50–15.00 | 1.25–7.75 | 1.37–7.25 |
| 18 | 46 | 4 | 75 | 68 | 32.75 | 3.75 | 4.87–32.75 | 1.37–4.25 | 1.25–11.00 |
| 19 | 31 | 3 | 75 | 66 | 40.00 | 31.25 | 1.25–40.00 | 1.50–13.75 | 1.50–31.25 |
| 20 | 56 | 6 | CT | 45 | 21.75 | 7.62 | 4.50–38.50 | 3.12–15.00 | 2.81–19.75 |
| $\bar{x}$ | 46.25 | 2.05 | – | 46.45 | 76.01 | 8.22 | 6.46–78.49 | 2.85–24.28 | 2.70–19.72 |
| SD | 9.22 | 1.36 | – | 21.44 | 83.35 | 7.15 | 8.73–81.79 | 1.77–16.53 | 1.79–12.50 |

CT – combination therapy (VEN in polypragmasy with sertraline 50 mg, trazodone 300 mg or mianserin 60 mg)

analyses, were used for data interpretation (14, 15). A matrix of the data with dimensions $n \times p$ was used, where $n$ is the number of rows, including women with MDD, and $p$ is the number of variables describing the studied subjects, such as age, hospitalization time and its multiplicity, initial and final cortisol level, the highest and the lowest cortisol levels and the difference between these values, mean concentration, median, stabilization of cortisol secretion at 8, 10 and 15 ng/mL during the whole hospitalization period and at different hospitalization phases. The matrix was $20 \times 22$ in dimension. In this study, a strategy without rotation of factors gave the best results in PCA, while in CA Ward's hierarchical agglomeration with Euclidean distance measure ensured the most interpretable results.

## RESULTS AND DISCUSSION

Analysis of the data compiled in Table 1 has shown that 20 women aged 46 ± 9, spent in the hospital about 46 ± 21 days. The patients treated with VEN in monotherapy were hospitalized for almost 48 days and in the case of women under combination therapy with VEN the mean hospitalization period was 42 days. The level of salivary cortisol was quantified every morning. The results have shown that the mean initial and final values were 76.01 ± 83.35 and 8.22 ± 7.15 ng/mL, respectively.

Statistical analysis of the data listed in Table 1 shows that the initial cortisol levels differ significantly from the final hormone levels ($Z = 3.9199$, $p = 0.0001$), but the dose of VEN had no influence either on the initial or final concentrations of the hormone. Moreover, statistically significant differences were found ($Z = 3.5893$, $p = 0.0003$) between the mean cortisol concentrations measured during 30% of hospitalization period and those determined during 60% of the period. In contrast to this, there were no significant differences in the mean cortisol concentrations between the values measured during 60% and 90% of hospitalization periods ($Z = 0.5973$, $p = 0.5503$). Furthermore, the dose of VEN ($F = 0.0919$, $p = 0.9124$) and time of hospitalization ($F = 1.9598$, $p = 0.2715$) did not have an impact on the mean concentrations of salivary cortisol during three periods of hospitalization. On the other hand, statistically significant differences in the time of hospitalization were found between mono- and combined-therapy ($Z = 3.9199$, $p = 0.0001$).

The results of statistical analysis show that the VEN therapy has an impact on the cortisol content in saliva of depressed women (statistically significant differences between the initial and final cortisol levels, and between the mean hormone levels during the first and second hospitalization periods). These results are compatible with the literature data (4, 6).

### Fluctuation of the salivary cortisol level

Day-by-day quantitation of the cortisol content in saliva of the subjects shows that a decrease in hormone level can take place in different ways. Detailed inspection of these data revealed that there are four different profiles of salivary cortisol changes. The first one presented in Fig. 1A relates to the patients whose organism responded to the treatment. The cortisol secretion decreased very quickly attaining the reference values. In saliva, the reference values for a healthy person are between 1 and 8 ng/mL (16). This level did not increase up to the end of hospitalization. The differences between cortisol concentrations in saliva as determined day-by-day surpassed few nanograms per milliliter. Hospitalization period of those patients did not exceed 25 days.

When the cortisol concentration decreased slower and the hospitalization exceeded 20 days, there were a few types of the cortisol secretion profiles. In the case of higher than 60 ng/mL initial cortisol levels, secretion of the hormone dropped in the first days of treatment achieving the reference values (Fig. 1B). In the next few days, the cortisol secretion increased and the amplitude of its concentration in saliva was larger than that during the stabilization period.

Another profile of the cortisol secretion is shown in Fig. 1C. When initial cortisol concentration was about 40 ng/mL, its secretion dropped very quickly attaining the reference values. In the case of patients staying at the hospital for more than 30 days, an increase in the hormone secretion occurred at about 30th day of hospitalization. After a few next days, the secretion declined and to the end of hospitalization the amplitude of cortisol level fell in the range of several or several dozen nanograms per milliliter of saliva. This revealed that the fluctuation of the cortisol secretion had a distinct impact on hospitalization time. This refers especially to patients with a large amplitude of cortisol secretion, typically several dozen nanograms per milliliter of saliva. In that case, the hospitalization period was longer than 30 days for the majority of depressed women.

About 20% of the studied patients treated with VEN in MT did not respond to the treatment. Therefore, five depressed women were treated in polypragmasy with VEN and sertraline, trazodone or mianserin. The mean hospitalization period was

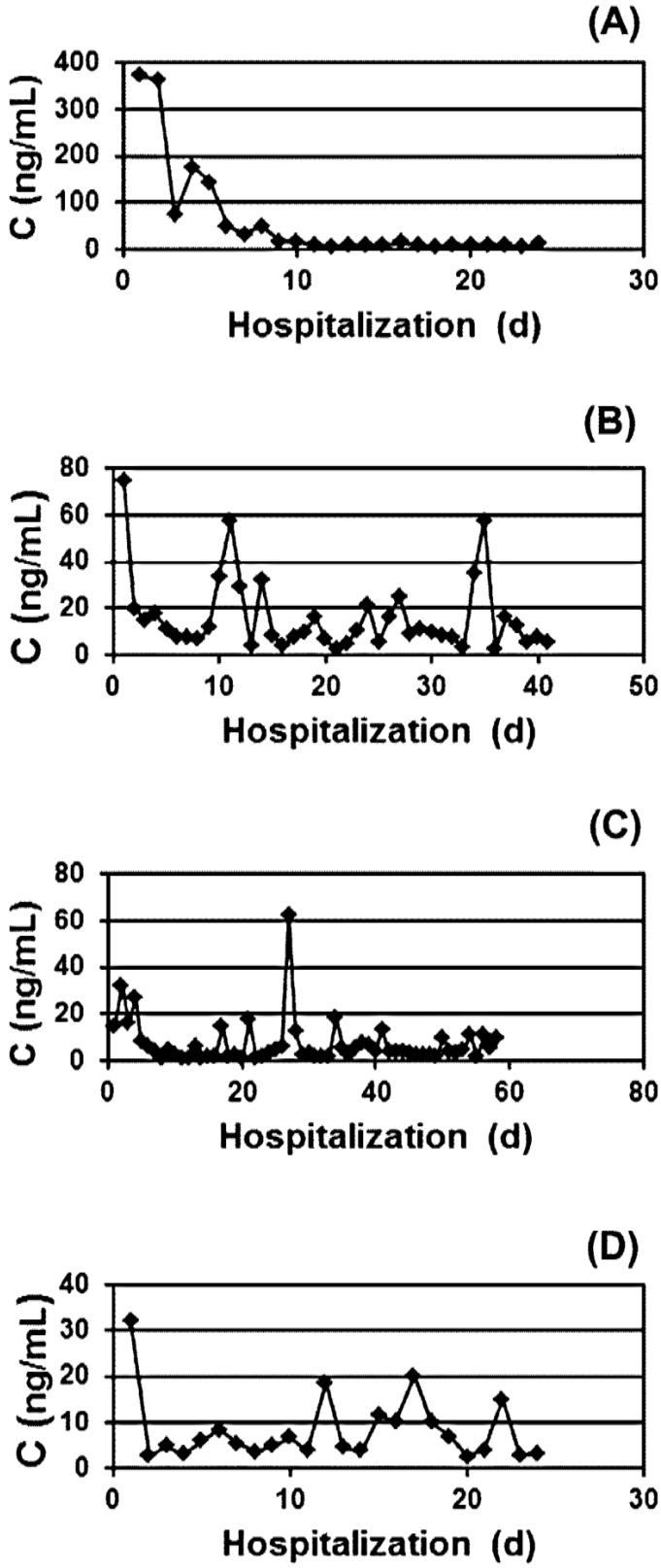


Figure 1. Profiles of cortisol secretion in women with major depressive disorder. Patients treated with venlafaxine: (A), (B), (C) – in monotherapy, (D) – in polypragmasy with sertraline, trazodone or mianserin

42 ± 9 days and was shorter than that for patients treated with VEN in monotherapy. Furthermore, differences between the salivary cortisol quantified day-by-day were lower, as is shown in Fig. 1D. This also refers to patients with a high initial level of cortisol, of about 100 ng/mL. The hormone concentration decreased in a few days and did not exceed 20 ng/mL up to the end of hospitalization. The second rise of the cortisol level at about 30th day of hospitalization did not occur as compared to patients treated with VEN in monotherapy.

To sum up, the impact of VEN therapy on cortisol secretion is diverse and depends on the state of health of the patient and also remitting of the treatment (5). This study supports these observations. In the case of a patient hospitalized for more than 30 days, a significant impact on cortisol level quantified day-by-day was noticed. Furthermore, the profiles of cortisol secretion revealed an increase in the hormone secretion at about 30th day of hospitalization. This rise of the cortisol secretion was not observed in the group of patients treated with VEN

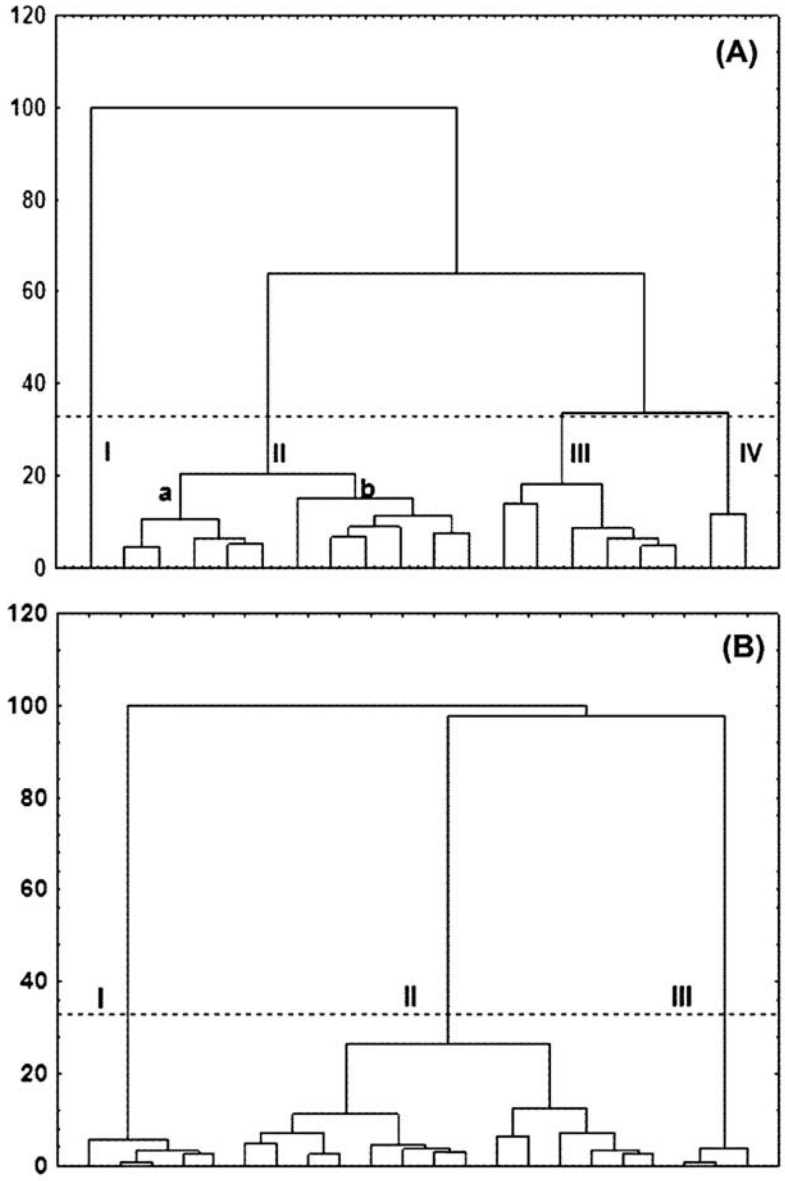


Figure 2. CA dendrograms illustrating the clustering of 20 subjects (A) and 22 variables (B), based on the data of Table 1

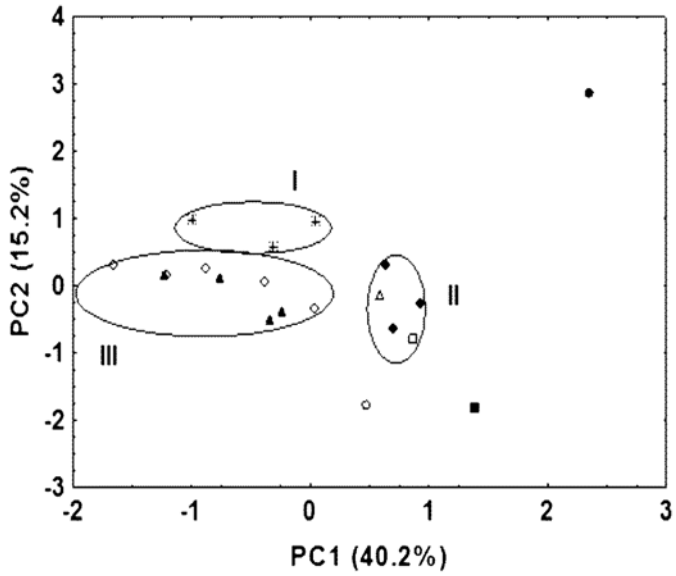


Figure 3. PCA score plot showing the grouping of 20 depressed women. Patients fall in Figure 2A in cluster I (*), cluster II (◇), and cluster III (◆). The youngest patient (●). Patients with high cortisol fluctuation (○), the shortest period of hospitalization (■), treated with 150 mg of VEN (□), treated in CT with mianserin or trazodone(▲), and treated in CT with sertraline (Δ)

in CT. This can be explained in terms of a relatively weak effect of VEN on the glucocorticoid receptor (GR) function to cause that the controlling of cortisol secretion is not strong. It also depends on the dosage of the drug (17). This study has shown that VEN used in combined therapy (CT) with other antidepressants, such as trazodone or mianserin, enhance the influence on GR. In the case of treatment with VEN and sertraline, which also affect rather weakly GR function, the fluctuation of cortisol level was larger. It is in a good agreement with the literature data on the cortisol secretion during SSRI therapy of depression (18).

**Multivariate analysis of the results**

Based on the day-by-day fluctuations of the cortisol concentration it was found that there are some different profiles of cortisol secretion as illustrated in Figure 1. To learn whether or not particular profiles differed widely in salivary cortisol level, a pattern recognition approach based on CA and PCA was used (14, 15).

Cluster analysis enables presentation in the form of a dendrogram, a way of grouping patients into particular clusters, with samples belonging to one cluster characterized by high similarity, and simultaneously, differing to the maximum extent from the other samples. As shown in Fig. 2A, twenty subjects with MDD were grouped into four clusters determined at 1/3 of the maximum distance using a Sneath's index criterion. A detailed analysis of the subjects found in these clusters revealed that variations in the amplitude of cortisol level in saliva had a vital impact on their classification. In the first cluster falls one patient only with a profile of salivary cortisol presented in Figure 1A. This subject is characterized by the highest initial concentration of cortisol in saliva (above 370 ng/mL) which decreased in the next few days to attain the reference value. As this patient differs strongly from the others, clusters II–IV are situated at a maximum distance from this one.

Clusters III and IV are the next ones which differ mostly from each other. Cluster III is formed by five patients treated with VEN in MT with profiles of cortisol changes shown in Figure 1B and one treated with VEN in CT. Almost all of them were hospitalized for the first time. In all cases the initial cortisol level was about 80 ng/mL. Those patients are characterized by enhanced fluctuation of the hormone secretion, which did not exceed 10 ng/mL. In the patient treated with VEN in polypragmasy with sertraline, the amplitude of cortisol level was substantial only at the beginning of hospitalization. On the other hand, cluster IV grouped 2 patients with a high initial cortisol level (above 150 ng/mL) which after a few days declined similarly as in the case of the patient from cluster I. The cortisol levels quanti-

fied day-by-day fell in the range of several nanograms per milliliter of saliva, especially at the end of hospitalization. Cluster IV encompassed two women, one with the longest period of hospitalization, 90 days.

Most of the patients studied (11) are located in cluster II, which can be divided into two subclusters, a and b, below 20% of the maximum distance. In this cluster are located four of the five subjects treated with VEN in polypragmasy. Three of them are in subcluster IIa, which encompasses patients with low amplitude in the hormone level, a few nanograms per milliliter during the whole period of hospitalization. The highest level of cortisol achieved in saliva of those patients did not exceed 20 ng/mL with a profile of cortisol secretion shown in Figure 1D. Subcluster IIb is formed by subjects with an enhanced fluctuation of cortisol secretion during the whole hospitalization period. In this case, the amplitude of cortisol level determined day-by-day was large, more than several dozen nanograms per milliliter. This subcluster encompasses patients with cortisol profile represented in Figure 1C, with initial cortisol level of about 40 ng/mL. The majority of these subjects were hospitalized because of the depression more than once.

The relation between 22 variables characterizing the subjects with MDD is shown in Figure 2B. CA revealed a strong correlation among multiplicity of hospitalization, highest and final cortisol levels, mean and median (cluster I). In this way CA connects multiplicity of hospitalization with salivary cortisol level. Another one was formed by variables relates to stabilization of the cortisol secretion at different levels during the whole hospitalization period and at different phase of the treatment, and also between hospitalization period and age of the patients. Both the initial and the lowest cortisol levels as well as the difference between the highest and the lowest levels are grouped in cluster III.

Another multivariate approach, PCA, enables interpretation of the data by reduction of dimensionality and adding clarity to presentation of the results. Because two first principal components (PCs), PC1 and PC2, explained more than 55% of the variability, a PCA score plot could be presented in the form of a two-dimensional plane (Fig. 3). In this case, the patients are grouped in three clusters. Cluster I is created by subjects with a high or very high level of initial cortisol concentration (between 84 and 173 ng/mL), but in this group the stabilization of cortisol secretion at the reference values has been achieved. Women with high initial cortisol concentration (about 100 ng/mL) and patients with high fluctuation of the cortisol during the whole hospitalization period are located in cluster II. The cortisol secretion profile for women located in this cluster is shown in Figure 1B and for one subject in Figure 1D. The woman marked with a white square was treated with 150 mg of VEN, whereas that marked with a white triangle was treated in polypragmasy with sertraline. Cluster III is created by patients with a cortisol secretion profile shown in Figure 1C. The initial cortisol concentration did not exceed 40 ng/mL. There are four out of five subject treated with VEN in polypragmasy (marked with a black triangle) with salivary cortisol profile shown in Figure 1D. The results are similar to those obtained by CA as presented in Figure 2A, cluster II.

It should also be mentioned that similarly as in CA (Fig. 2A, cluster I), an outstanding patient marked with a dot is located separately in the right corner of the PCA plot. Furthermore, the next two subjects are also located separately. The first marked with a black square is characterized by a very short hospitalization period and decreasing cortisol secretion during the first days of treatment. The other (white point) demonstrated the high fluctuation of hormone secretion.

The most significant impact on classification of the subject according to the PC1 axis had the stabilization of cortisol secretion at different levels during the whole hospitalization period and after elapsing of 30% of the period at the levels of 8 and 10 ng/mL of cortisol. The mean concentration was important as well. According to PC2 axis, the initial and the lowest cortisol levels, and also stabilization of cortisol secretion at 15 ng/mL after 30 and 60% of the hospitalization period were the most important factors for classification of the patients.

## CONCLUSIONS

Based on the results obtained in this study, it can be stated that the VEN used in the depressed women therapy in MT and CT with sertraline, trazodone or mianserin has an impact on the cortisol secretion, the secretion has been suppressed. This can be supported by concentration of the hormone at the end of hospitalization, i.e., by the mean initial and final salivary cortisol levels which were 76.01 ± 83.35 and 8.22 ± 7.15 ng/mL, respectively. Furthermore, based on the highest and the lowest cortisol levels during different periods of hospitalization, it can generally be concluded that fluctuation of the hormone secretion decreases during hospitalization. Thus, it can be stated that there are some different profiles of cortisol secretion as shown in Figure 1.

These findings also demonstrate that VEN used in CT significantly reduced the fluctuation of cortisol concentration as quantified day-by-day during the whole hospitalization period. For instance, for the women under CT therapy with VEN the mean hospitalization period was 42 days, whereas those treated with VEN in MT were hospitalized for almost 48 days. In conclusion, the combined therapy distinctly reduces the time of cure.

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## GENERAL

# THERAPY REMINDER MESSAGE FOR HUNGARIAN PATIENTS WITH TYPE 2 DIABETES

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**Abstract:** Adherence is an essential element for the optimal treatment while it is closely related to the outcome of chronic diseases. Non-adherence rate is really high; increasing the adherence is an important task for health-care professionals in order to treat the morbidity and co-morbidity and to prevent complication. The object of this study was to find a method which can improve patients' adherence in type 2 diabetes. A one year long prospective study was carried out from 2013 March. One hundred thirty one diabetic patients joined the survey, 66 in the control and 65 in the intervention group. Patients in the intervention group received a reminder SMS three times a day to take their medicine. Glycated hemoglobin and blood glucose level was measured regularly. The patients' parameters and the difference of the values were examined. No significant difference was found between the control and intervention groups. In the survey significant results was found in the final glycated hemoglobin values. During the time of the research, the patients became active participants of their therapy. Due to this active co-operation, positive results could be achieved. Based on the results, a decision making support module should be implemented to the existing medical information system to improve the therapeutic adherence of wide range of patients.

**Keywords:** chronic diseases, adherence, pharmaceutical care, glycosylated hemoglobin, reminder SMS

Chronic diseases are the leading mortality cause with more than 60% of all death, according to the WHO (World Health Organisation). Patients with chronic diseases generally suffer in polymorbidity. The success of the complex therapy highly depends on how the patients adhere their medication. To achieve good adherence is the main goal of health-care professionals in order to decrease mortality in patients with chronic diseases. Pharmacist can influence many factors which improve adherence. Besides guidance, adherence must be followed up because in chronic diseases the poor adherence increases with long duration and the complexity of the treatment. The highly prevalent diabetes mellitus and its co-morbidity with complications is becoming a major public health problem worldwide. In Hungary the prevalence of diabetes is 5-5.5%, this means 500.000 patients. Approximately 90% of all cases belong to type 2 diabetes. Achieving optimal blood glucose level and preventing the chronic complications are the goals of the medical therapy. Complexity of this heterogeneous disease needs a complex approach and treatment in which the patient is an active participant, too. The result is greatly influenced by how the patients take their medicine, so the adherence is an important factor which has to be examined for the successful therapy. In developed countries the adherence to medical therapy is approximately 50%, in long-term medication this rate is considered to be low (1). There are several statistics showing this result and the adherence is far from optimal, therefore reducing effectiveness of the medical therapy and treatment results (2, 3). A Hungarian study from 2007 published adherence results to diet (76.8%), physical activity (33.8%), self-monitoring of blood glucose (81%), drug purchasing (20.4%) and drug taking 52.1% (4). Further research found adherence rate between 47.9% and 49.2% to oral antihyperglycemic drugs. There was significant difference between male and female patients, women had a significantly better ($p < 0.001$) adherence rate than men, 51.3% *vs*. 45.5% (5).

In diabetic patients the HbA1C (glycated hemoglobin) measurement is a basic test in

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glycemic control, while it represents average blood glucose over three months. The duration of diabetes and the complications are significantly associated with the glycated hemoglobin. In T2DM (type 2 diabetes) 1% increase of HbA1C poses 15-18% relative risk of cardiovascular disease (6). In the case of AMI (acute myocardial infarction) 14% reduction was found in association with 1% reduction of HbA1C 1% in UKPDS (United Kingdom Prospective Diabetes Study).

Correlation is observed between adherence and HbA1C: 10% increase of adherence decreases HbA1c with 0.1% (7), good adherence to drug therapy is associated with decreased mortality and with positive health outcomes. Therefore, the primary aim of this study was to study the effectiveness of therapy reminder message on the patients' adherence in type 2 diabetes and the consequent specific disease parameters.

## EXPERIMENTAL

A prospective study was carried out in patients with T2DM from March 1, 2013 to May 31, 2014 in Miskolc, Borsod-Abaúj-Zemplén county. Seven general practitioners took part in the survey. Patient recruitment was performed 2 months long with the help of their general practitioners. The following criteria had to be fulfilled for the patients who took part in the survey:

– diagnosed type 2 diabetes
– oral antidiabetic therapy
– cell phone user
– regular medial check (monthly)
– age (30-65 ages)

Each patient had a personal file containing: name, age, medication, and blood glucose and glycated hemoglobin. During the survey, regular glycemic controls were made. The blood glucose level measurement was performed monthly by the general practitioner in each visit. The glycated hemoglobin was measured among laboratory conditions every three months. The participants were divided into two groups: A and B. To divide the participants into the intervention and control groups we used the RAND() formula of Microsoft Excel. The participants were put into an Excel worksheet and with the RAND() formula a random numbers to each one of them were assigned. They were sorted in ascending order based on the random numbers assigned to them and chose the first half of them to be in the intervention group, and the second half of them to be in the control group.

Patients belonging to group A got a reminder message on their cell phone three times a day (08 a.m., 02 p.m. and 08 p.m.) to take their medicine. Descriptive statistics for the groups are summarized in Table 1.

Patients belonging to group B did not get any reminder SMS. Glycated hemoglobin levels were compared between the above groups. The aim of the study is to improve adherence by reminding patients to take their medicine regularly, in order to achieve optimal Hba1C level.

The study was authorized by the Regional Ethics Committee (RKEB:01-01-2010). All patients gave written consent to participate.

Table 1. Descriptive statistics for groups A and B.

| Patients | Number | Minimum age | Maximum age | Mean | S.D. |
|---|---|---|---|---|---|
| SMS group (A) | 65 | 40.00 | 82.00 | 59.9385 | 8.71937 |
| Control group (B) | 66 | 40.00 | 75.00 | 60.2154 | 7.89084 |

Table 2. The summarized group statistics.

| | Adherence group | N | Mean | S.D. | S.E. Mean |
|---|---|---|---|---|---|
| HBAFIN | SMS | 65 | 6.7015 | 1.06778 | 0.13244 |
| | control | 66 | 6.6561 | 1.19062 | 0.14656 |
| BLODGLU- | SMS | 65 | 7.4200 | 1.79463 | 0.22260 |
| FIN | control | 66 | 7.4821 | 1.82328 | 0.22443 |

S.D. = standard deviation, S.E. = standard error

Table 3. Deviation values of the glycated hemoglobin difference.

| | N | Mean | S.D. | S.E. | 95% confidence interval for the mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | | |
| SMS | 65 | -0.0585 | 0.84167 | 0.10440 | -0.2670 | 0.1501 | -2.30 | 2.40 |
| Control | 66 | -0.4045 | 1.11265 | 0.13696 | -0.6781 | -0.1310 | -3.40 | 3.10 |
| Total | 131 | -0.2328 | 0.99896 | 0.08728 | -0.4055 | -0.0602 | -3.40 | 3.10 |

S.D. = standard deviation, S.E. = standard error

Table 4. Initial and final glycated hemoglobin values.

| SMS (A) group | Initial HbA1C | Final HbA1C |
|---|---|---|
| Male | 6.69 | 6.765 |
| Female | 6.851 | 6.677 |
| Control (B) group | | |
| Male | 7.019 | 6.644 |
| Female | 7.100 | 6.668 |

Statistical comparisons were performed with two sample t-test and ANOVA.

The figures/results were aggregated with Excel.

**Technical information**

A web based application was used to provide the research with IT support. The application is able to send SMS to the patients to remind them to take their medicine used in the diabetes therapy. Administration of the patients' data included in the research could be worked out on the web based user interface of the application. It can be customized which patients should get a reminder SMS in the morning, at noon and in the evening. Also the text of the messages can be customized. Doctors can log in to the system and enter diabetes related data of their patients; the system stores this data historically. This feature enables us to handle research data effectively when creating statistics and drawing conclusions.

**RESULTS**

The study was designed with 140 diabetic patients, after one year, 9 patients dropped out. From the 131 patients 70 were male and 61 were female, average of 60 years. The average age was 59 years in the intervention group, 60 years in the control group. There were 38 male and 27 female patients in the intervention group. In the control group, there were 32 male and 34 female patients.

Oral antidiabetic therapy was a recruitment criterion. The OAD medication was recorded during the study; 45.67% of the patients had OAD monotherapy: 27.1% biguanid (metformin), 12.3% sulfonylurea, 4.9% thiazolidindion and 1.2% a-glucosidase inhibitor.

Combination therapies:

Biguanid (metformin) + sulfonylurea: 43.2%

Biguanid (metformin) + thiazolidindion: 3.7%

Sulfonylurea + thiazolidindion: 2.4%

Biguanid (metformin) + sulfonylurea + thiazolidindion: 3.7%

Biguanid (metformin) + sulfonylurea + $\alpha$ glucosidase inhibitor:1.2%

The two groups were intervention group A (initial HbA1C value 6.76%) and control group B (initial HbA1C value 7.06%).

During the survey the glycated hemoglobin and the blood glucose levels were measured.

There was no significant difference in the final value of glycated hemoglobin (HBAFIN) between the groups ($p = 0.212$).

Neither were there significant differences in view of final blood glucose value (BLODGLUFIN) ($p = 0.845$) as it can be seen in Table 2.

Table 2 shows the final glycated hemoglobin (HBAFIN) and final blood glucose (BLODGLUFIN) values.

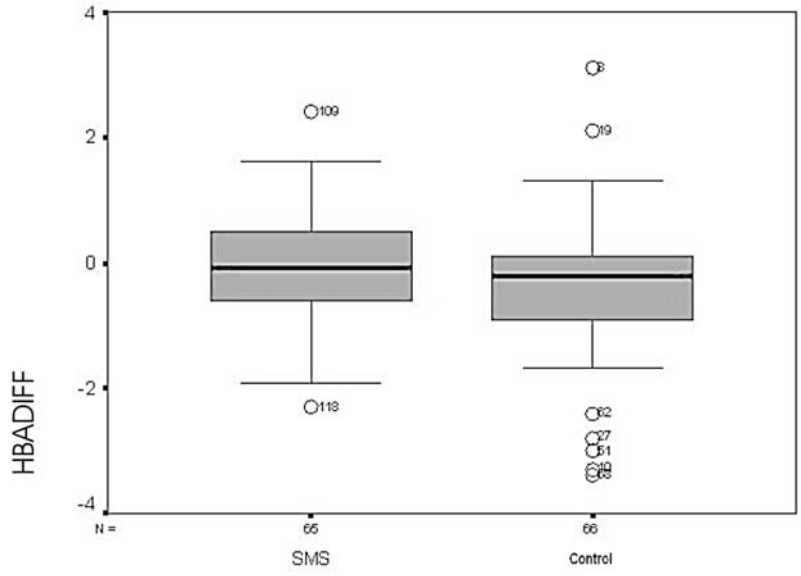


Figure 1. Difference in glycated hemoglobin levels of the SMS (adherence group) and control group

Similarly to other studies in these topic, no statistical differences were noticed between patients receiving messaging interventions compared to those who received only the usual medical care (8).

In spite of the fact that in the SMS group the HbA1c reduction was significantly lower, the deviation was lower, too, and the therapy result became more stable than in the control group (Fig. 1).

The box plot giving median, quartiles and outliers can be seen in Figure 1.

Table 3 includes deviation values of the glycated hemoglobin difference (HBADIFF).

The initial and final values of glycated hemoglobin were also examined (HBADIFF). Significant difference was found ($p = 0.047$) between the initial and the final values of glycated hemoglobin levels.

The measured blood glucose level did not show (BLODGLUDIFF) significant results ($p = 0.856$). There was no difference between the results found in the case of male and female patients. Table 4 summarizes the initial and final glycated hemoglobin values.

Improving adherence especially in patients with chronic diseases is a great challenge for the healthcare professional. Normoglycemia is the key to achieve in patients with type 2 diabetes reduction of complications and co-morbidity. The benefit of the control of glycemic parameters is evident (9, 10). While glycated hemoglobin and medical adherence are associated (11), the healthcare professionals have to find a way to solve the problem of non-adherence for the successful long-duration therapy. Web and phone based technology designed by experts can be the solution and a theme for adherence-improving researchers (12-16), because it was found that patients taking part in the message reminder program have a much higher adherence concerning their medication, than those patients who do not take part in the message reminder program (17, 18). In spite of that, this study did not confirm the benefit of reminder messages; further research is needed with larger number of patients and performed for a longer term.

## DISCUSSION AND CONCLUSION

Reduction of HbA1c could be achieved after 1 year in both groups and this was the goal. The reason for this result was that the patients were active participants in their treatment.

The task of pharmacists and pharmaceutical care is to find the proper way to cooperate more closely with patients concerning education, therapy monitoring control, adverse effects and adherence to achieve a successful medical therapy. This study shows that with proper attention the healthcare professionals can influence the patients' healthcare out-

comes when the patients are ready to cooperate with them.

In Hungary, the codified pharmaceutical care provides the patients who have chronic diseases the opportunity to learn about their disease, medication, measuring and controlling their blood glucose and lipid levels and getting answers for their questions. Practice implications of the above study is that the applied therapy reminder message was effective in a way that the deviations of the key parameters (glucose level and glycated hemoglobin) were lower in the test group compared to the control group, although their absolute values did not change significantly. The latter indicates that the cost-effectiveness ratio of the method is not sufficient enough in the case of patients showing acceptable therapeutic adherence. Based on the results, a decision making support module should be implemented to the existing medical information system to improve the therapeutic adherence of wide range of patients.

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# ELECTRONIC PRESCRIPTION SERVICES SYSTEM IN GREECE – PILOT STUDY

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**Abstract:** The aim of the work is to provide an overview of Electronic Prescription Services in Greece, to illustrate a detailed description of the Greek Electronic Prescription Services system, to evaluate how it has changed, the way public healthcare system works, to analyze major benefits and potential problems faced. The self reported questionnaire was distributed to the three main stakeholders in the medication prescription process: doctors, patients and pharmacists. The collected results have been processed in a spreadsheet application. The results have been analyzed individually but also in comparison with relevant results from each group of people. It has been identified that the major aspects of interest are the ease of use of the new system, the safety that the new system introduces regarding medication and dispensing errors, the level of service that the patient receives, the administrative and back-office workload and how it is managed, the economical aspect as far as the public expenditure is concerned but also the costs for the professionals themselves and finally, the technological aspect and how it affects the users. The new system has been able to overall improve the process of drug prescribing from the safety perspective (less medication errors), level of service (more interpersonal communication and less administrative overload with the patient), economical aspect (mainly as far as the public expenditure is concerned) and back office administration. As any other new system, it has been also identified that most users struggle to familiarize, spend more time especially at the beginning while trying to learn how to use it. There is room for improvement for the system itself but mainly for the organized, well-structured and extensive training of the users in this continuously changing and dynamic environment that healthcare is.

**Keywords:** electronic prescription, electronic prescription services system, benefits, drawbacks, ease of use, safety, economic aspects, technology, administration, level of services

The term "Electronic Prescription Services" (EPS) refers to the production, distribution and control of prescriptions and referrals for medical procedures, using technology and/or computers and telecommunications, in a way that ensures the validity, security and transparency of information handled. In its full range it supports processes of creation, execution, management, monitoring, validation and payment of prescription drugs and medical procedures at all points of interest (health center, doctor's office, clinic, hospital, pharmacy, diagnostic laboratory, etc.) and provides important capabilities for monitoring, research and analysis for all stakeholders. In order to replace the existing legacy systems of handwritten prescriptions and install a modern high-tech online infrastructure, a lot of complex interventions are required: a sufficient maturation period, learning period, support and maintenance. Major issues had to be resolved such as the complexity of the current procedures, lack of legislation framework, lack of up-to-date patient and medical records, the large number of involved stakeholders, their diverging interests, the protection of personal data, the geographical dispersion of points creating and executing recipes, and so on. Nevertheless, the new EPS system is currently one of the largest IT infrastructures operating in Greece, executing hundreds of thousands of transactions on a daily basis. It is a big bet in order to improve public health and reduce costs involved, especially during a fragile economic environment severely hit by the financial crisis in the country (1).

The economic crisis and the requirements for structural changes in all aspects of public administration brought a new era of reforms in the country as well as the healthcare system itself. A new system was required in order to deal with several new demands that the new fragile financial balance brought. The system should be designed to record and control the movement of medical visits / pre-

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scriptions / referrals, settlement and payment, and was expected to bring benefits to protection of the health and reduce the suffering of the patients, reducing the cost of primary care by limiting cases of over-prescribing, promoting the use of generics and providing enhanced scientific support to health professionals, policymakers in health and medicine. The objectives of the project also included the streamlining of prescribing processes, their execution and control, the development of an open system incorporating the international best standards and practices, to give third parties the possibility to develop innovative services associated with the care providers and health system in general. According to e-Government Social Insurance, the project's success is judged primarily on the extent of use and the use of specialized capabilities, aiming mainly in the promotion of health of the insured patients (2).

The Electronic Prescribing process can be defined as the electronic creation and transmission and processing of a medical prescription instead of using traditional paper handwritten prescriptions. The doctor or the prescriber in general can electronically (using computers and the internet) create the prescription (the first or a repeated copy of it) and possibly transmit it directly to the pharmacy through a secure channel *via* the internet. In other case, the electronic prescription is created and stored in the "cloud", however, it is not transmitted automatically to the pharmacy but the patient is delivering it to the pharmacy in person. EPS is meant to reduce the risks and errors associated with traditional prescription script writing. It is also one of the major steps towards the generation of electronic medical records. The sharing of medical records and information regarding the prescription history or medication history of a patient can help the healthcare providers by giving them a robust decision making system (3).

The main functions that should be carried out by the EPS system are creating an up-to-date list of medicines the patient is taking; printing, selecting and transmitting prescriptions, making all the necessary safety checks, integrating decision support systems; checking patients' current medication list and medication history information within the practice; giving information related to the availability of lower cost, therapeutically appropriate alternatives; working with an existing medication within the practice; prescribing or adding new medication and selecting the pharmacy where the prescription will be filled; sending the information to the Transaction Hub, where information on the patient eligibility, formulary, and medication history/fill status is sent back to the prescriber; giving information on formulary or tiered formulary medications, patient eligibility, and authorization requirements received electronically from the patient's drug plan; patient-specific information capabilities (e.g., current patient medication list, access to patient historical data, patient identification); system integration capabilities (e.g., connection with various databases, connection with pharmacy and pharmacy benefit manager systems); learning and educational capabilities (e.g., patient education, provider feedback) (4-6). The basic components of the EPS system are the doctor or prescriber, central server infrastructure, pharmacy with implemented electronic prescribing software and pharmacy assistive software.

It's clear that EPS system can have a major and positive impact on clinical practice and patient care. Experience shows that EPS systems are most effective when front line staff have been involved at every stage of their design, development, testing and

Table 1. The most common prescription errors in handwritten prescriptions.

| Type of error | Appearance [%] |
|---|---|
| Dispensing different medicine than the one prescribed | 66.0 |
| Drug barcode unique identifier different that the medicines list | 9.9 |
| Number of drug packages dispensed different than the prescribed | 3.8 |
| Not correct processing of repeated prescription | 3.7 |
| Dispensing of more than 2 packages even though not allowed according to instructions | 3.3 |
| Dispensing drugs that exceed the maximum allowed value without authorization | 3.1 |
| Various other errors | 10.2 |

Table 2. Key fields of national level e-Health activities in the EU 27 countries, 2007 and 2010.

| Reported e-Health activities | Total 2007 e-Health ERA* | Total 2010 e- Health Strategies | Δ |
|---|---|---|---|
| Legal activities | 14 | 22 | 8 |
| Evaluation | 5 | 21 | 16 |
| EHR Patient Summary | 27 | 27 | 0 |
| e-Prescription | 16 | 22 | 6 |
| Tele-health | 23 | 27 | 4 |
| Patient ID | 24 | 26 | 2 |
| Professional ID | 13 | 22 | 9 |
| Citizen card | 22 | 25 | 3 |
| Professional card | 7 | 18 | 9 |
| Standards (technical/semantic) | 19 | 27 | 8 |

* ERA = European Research Area in e-Health

implementation (7). More specifically, the benefits for the patients as well as the prescribers include better safety and quality of care for the patient, reducing delays and time spent for clarifications, alerts and warnings are provided at the point of care, access to medical records, automating the prescription renewal request and authorization process, enhancing medication compliance, benefits from lower cost drug substitutions, improving prescriber mobility – geographical dispersion and better drug surveillance (8).

Despite the fact that EPS is able to increase speed and efficiency in all the processes, the extended use of such a system has also some important drawbacks. Some of the difficulties that have to be overcome are resistance to change, user training and seminars, hardware and software selection, erroneous alerts, integrity of data input, security and privacy, system downtime (9-11). The previous handwritten prescriptions system introduced several risks and errors as quantified in Table 1, using data from the major social security fund IKA (Social insurance in Greece) in 2003 (12).

In order to cope with those problems and make a giant step towards the reform of the Greek healthcare system, a new fully electronic and state-of-the-art system was introduced in 2010. The EPS is a complex system that supports all doctors, clinics, hospitals, pharmacists and patients in a big and geographically dispersed country like Greece. It is reported that by June 2012 more than 10,500 pharmacists and 37,500 doctors were using the new EPS (13). Based on the data supplied by the operator IDIKA S.A., the EPS system is currently used by the majority of the healthcare providers for supporting almost all medication prescriptions in the country, making it the largest on-line system in Greece today (14).

The EPS system will manage most economic and medical evidence under a high security regime that is dictated by its sensitive character. The procedures and functions are dictated by the institutional and regulatory framework and the rules and practices of good prescribing and referral. Still, it provides the entire Management Information System (MIS) that is required in a reformed modern healthcare system. In terms of functionality, the system should cover all the functions involved in the process of medical visit / prescription / referrals of laboratory tests, and monitor their status until the payment of the final beneficiaries (reimbursement).

Today, most European countries surveyed have more detailed documents published outlining concrete policies / strategies on electronic health goals, measures and/or implementation objectives and achievements. Based on such documents, the overview in Table 2 identifies key fields of national level e-health activities and demonstrates the considerable increase recorded between 2007 and 2010 (15).

The aims and objectives of the work were to provide an overview of EPS and how they apply to the Greek healthcare services market. Work evaluates its acceptance under the perspective of the 3 major stakeholders: pharmacists, patients, doctors / prescribers. It analyzes and discusses how it has changed the way public healthcare system works under 6 major pillars: ease of use, safety, economy, administration, level of service and technology. It discusses

major benefits and potential problems faced from the overall perspective of the healthcare professional or patient, and how improvements can be made.

## MATERIALS AND METHODS

The methodology followed, was based on a survey using structured questionnaires according to the type of responders (16). The pharmacists, patients, doctors / prescribers were reached personally or through telephone interview in the city of Athens, Greece, municipalities of Helioupolis, Argyroupolis, Glyfada, in the time period 17$^{th}$ November 2013 through 17$^{th}$ March 2014.

## RESULTS

The questionnaires have been delivered to a segment of pharmacists (29 pharmacist questionnaires), patients (56 patient questionnaires received) and doctors / prescribers (11 questionnaires). The results were gathered and consolidated using relevant spreadsheet software in order to generate comprehensive outcomes (MS Excel, SPSS 13) (17).

### Group - pharmacists

Demographic data of pharmacists: 13 men, 16 women; 11 age 18-34, 15 age 35-64, and 3 age 65 and more.

***Ease of use - usability perspective and ease of use***

The vast majority of the pharmacists (93%) find it easier to read the electronic prescription compared to the older handwritten form, 86% find it faster to execute an e-prescription compared to the past, 76% are able to service the patients faster using the e-prescription system and the customer throughput (customers per hour) have increased. More than 90% of pharmacists find it easier to choose among the appropriate drugs in order to best suit with the patients needs.

***Safety - e-prescription system from the safety perspective***

Ninety percent of the questioned pharmacists feel totally safer regarding any possible medication error, the vast majority of the pharmacists are able to confirm that they feel much safer that they will receive the correct medication since they know that the errors are far less probable to happen. Almost 100% of the pharmacists were totally confident that they are doing less dispensing mistakes now with the e-prescription system compared to the old handwritten prescription forms.

***Administration - the administrative and back office work***

Most of the pharmacists find it easier to do the back-office work and the majority (71%) of pharmacists find it faster to perform these back-office monthly tasks.

***Economy - economical aspects of the new system***

Eighty percent of pharmacists questioned believe that the overall health spending has been rationalized now with the e-prescription system and 72% believe that their cost of doing business (the costs associated with the processing of an e-prescription) has increased instead of decreasing.

***Technology - technological perspective***

Many of pharmacists (45%) not to feel very confident using the new system so far because all the new IT systems were introduced at a very short period of time and pharmacists did not have the time to attend seminars and other training necessary. In the case of internet outage, or computer malfunctions, almost all of the pharmacists questioned (96%) are certain that they would not be able to do their job properly. Most of the individuals questioned (66%) found it difficult to get familiar and learn how to use the new system.

### Group - patients

Demographic data of patients: 29 men, 27 women; 16 age 18-34, 21 age 35-64, and 19 age 65 and more.

***Ease of use - usability perspective and ease of use***

Almost 3/4 of the asked patients (71%) say that it is not faster for their doctors to prescribe them their medication now with the new e-prescription system. Each newly introduced system and process has a learning curve (18), which means that it takes some time for the users to learn how to use the system, gain experience and then start to be more productive (produce more prescriptions per hour). Eighty-six percent of the patients can tell that their pharmacist is able to execute the prescription and dispense the medicines much faster than in the past.

***Safety - e-prescription system from the safety perspective***

Fifty-five percent of patients think that it is better for them to be able to read the prescriptions themselves. The vast majority (98%) of the patients are feeling much more confident that the pharmacist will prescribe the correct medication now with the computerized system and this is because they are aware that IT systems minimize the errors.

***Level of service - level of service changed with the newly introduced system***

Almost all of the patients (over 90%) say that they are satisfied with the amount of time they spend in the pharmacy and are being serviced fast enough. Most of the patients (more than 60%) find the level of service they receive increased compared to the past.

***Economy - economical aspects of the new system***

The majority of the patients (77%) answered that they are not spending less money for their monthly medication prescriptions. Most of the questioned patients believe that the newly introduced system has helped the insurance funds save money. Some patients believe that they receive the correct amount of medication they need, some patients believe that they receive probably less medicines than they need.

**Group - doctors / prescribers**

Demographic data of doctors / prescribers: 6 man, 5 women; 4 age 18-34, 5 age 35-64, and 2 age 65 and more.

***Ease of use - usability perspective and ease of use***

It is interesting that 64% of the doctors asked, find it more difficult to prescribe an e-prescription compared to the past. This is mainly because the system is relatively new and the users are not familiar with it yet. Moreover, most of the doctors were not properly trained. More than 50% of the individuals asked, find it slower to complete an e-prescription compared to the old handwritten form.

***Safety - e-prescription system from the safety perspective***

The majority of the doctors asked (82%) believe that the errors during prescribing have decreased significantly now with the new system and more than 90% of the doctors are confident that the pharmacist will eventually dispense the correct medication.

***Economy - economical aspects of the new system***

More than 80% of the prescribers believe that the overall healthcare publics spending for the medicines prescription have been rationalized now with the new system. The new system allows the central administration and authorities of the Ministry of Health to monitor the prescription volume on a monthly (and even daily) basis and forecast accordingly. Almost 90% of the doctors said that their cost-of-doing-business has increased now with the new system. This is since the doctors had to purchase the necessary equipment (computers, printers, etc) without any funds from the state.

***Technology - technological perspective***

Almost 45% of the doctors asked, feel confident to use the EPS systems for their daily tasks while the rest don't feel so confident. All of the individuals asked, find it impossible to operate their business without computers and the internet. Most of the users (64%) found it very difficult to learn how to use the new system mainly because they did not receive the proper training. The newly introduced system is quite complex and the doctors had to learn how to use it in a very short period of time (a couple of months only).

## DISCUSSION

The results and discussion of the three types of questionnaires, for the pharmacist, the patient and the doctor are based on the sections: ease of use, safety, administration, economy, level of service and technology (19).

From the ease of use perspective, even though the doctors / prescribers find it somehow more difficult to create the e-prescription, the pharmacists find it easier to execute the prescription. This shows that the most effort has been made to design a straightforward system for the final user (pharmacist) while there is room for improvements at the prescriber's end. Probably, some extra automations or filters (e.g., automatic suggestions based on previous month's prescription for the same patient) could be implemented to help the doctors prescribe easier. Time-wise, again the pharmacists are able to execute the prescription much faster with the new e-prescription system compared to the past. Moreover, they are able to service more patients per hour, decrease the time spent on the administration tasks and increase the interpersonal time with the patient, explain them how to use the medicine, give them advice, listen to the patient's problems and suggest solutions. The impact on staff and professionals is crucial in most implementations and this has allowed the staff more flexibility over planning their time (20). The patients themselves are confirming that their waiting time in the pharmacy has decreased and they feel that the level of service is better than it used to be. This is mainly because the pharmacist has more time to spend with them, they are able to consult the pharmacist for other issues they may have, it is much more easy for the patient to read the prescription themselves and then discuss

with the pharmacist on whether they would choose to get a generic medicine, which one, how much money would this cost, etc. An analysis of a similar system for the NHS in the UK showed that the average time spent per prescription has been decreased from 70 seconds (paper version) to 53 seconds (electronic version) (21). A demographic analysis of the results also shows that younger patients are more certain that the level of service has increased with the new e-prescription system while younger pharmacists and doctors find it easier and faster to use all the new systems. This makes sense because younger professionals are much more confident using new computerized systems compared to their older colleagues.

Looking at the results from the safety perspective, one can easily tell that the new electronic system has made a significant breakthrough regarding safety. Prescription and dispensing errors have been decreased to a minimum because the doctors and pharmacists are employed with an arsenal of computerized safety mechanisms to help them do their job: provide the right medicine to the patient in a timely and convenient way. The doctors and pharmacists, aided by their computer systems are able to compare with previous medication that the patient took, keep, search and find historical data of previous prescriptions (22). This decision support system, helps the health professional choose the right medication for the patient. Then, in case the pharmacist accidentally tries to dispense the wrong e.g., dosage form, the computer will automatically generate an alert. It is therefore commonly accepted by all three basic stakeholders (pharmacists, patients and doctors) that the medication errors have significantly decreased, while the level of confidence that the patient will receive the appropriate medication at the right cost has increased. These findings are in line with most implementations of electronic prescription systems where medication errors have also been significantly reduced as far as prescription errors and pharmacist interventions are concerned, with more than 62% reduction (141 errors before compared to 53 errors after) (20).

From the administration perspective, the new e-prescription system together with each pharmacy's Point of Sales Software system has changed the way the back-office administration tasks at the end of the month are being made. For the younger professionals it is easier to consolidate the data, make and print summary tables and lists of the monthly prescriptions. These data are being submitted at the end of each month to the public insurance fund in order for the pharmacy to get reimbursed. In the past, all these data had to be handwritten in long sheets of paper and all the calculations had to be made by hand. As one can easily understand, this was a very time consuming process which also included many calculation mistakes if a digit was misspelled or miswritten. With the introduction of the computerized system, all these have been made much more straightforward. However, since the users are not yet familiar with the new systems completely, the older professionals don't seem to realize how they would benefit from all these at some extend. It is more than certain however, that as time passes, the users will find it easier and faster to perform all these tasks using the computer.

From the economic perspective, all stakeholders agree that the new system will be able to rationalize the public expense on health care and medicines in particular. Over the past years, extensive cases of fraudulent over-prescription have been identified and this is mainly the reason that the national healthcare expense had been at the all-time high before the economical crisis of 2010. Healthcare expenditure cuts together with all the other major national reforms were necessary and the electronic prescription system was one of the major infrastructures developed towards the rationalization of the expenditure. Pharmacists, doctors and patients, believe that this is happening as it was originally expected. The system makes it very easy now to identify any abnormal deviation from the average daily or monthly prescription volumes and auditors can easily target the prescribers responsible for those abnormal or strange activity. On the other hand, for the health professionals themselves it appears that the newly introduced changes have increased their monthly cost of doing business mainly because all the necessary equipment had to be purchased by the professionals. This perception is in line with the financial findings from the period of operation of the EPS in Greece, according to the officials: "The average cost of each prescription has been reduced by 30% from January 2012 until today, resulting in savings of 30 million euro per month, or an annual 300 million euro cost saving" (23).

From the technological perspective the new system made it impossible for the doctor or pharmacist to operate without the use of computers or internet. Moreover, the professionals were actually forced to start using the system in a very short amount of time even though it had problems at the beginning. This is the case in other EPS systems (e.g., in the UK) where users also appeared frustrated during the problem of the first period of operation

(21). All these problems together with the difficulties that older (usually) professional have use the computer efficiently and fluently, decreased the level of confidence that the professionals will manage to make it through this labyrinth of changes. The main reason that this happened is the fact that there were no official trainings or seminars (probably because of time limitations or insufficient budget) for the users, pharmacists and doctors. Moreover, many of the professionals who didn't feel quite comfortable with the IT systems maintenance had to employ technical support or even secretarial support to help them with the extra workload. This is closely connected with the previous section analysis that showed an increase of the cost-of-doing-business. However, since the younger professionals appear to be more confident with the new technology, it can be said that in the future these problems will become less and less.

## CONCLUSION

In the days of the economical crisis in Greece, the national public heath expenditure has been one of the major discussion fields and very high at the political agenda. It was more than obvious that reforms should be made in order to rationalize those expenses. One of the major reforms that were introduced in the country was the Electronic Prescription System operating at the national level. The whole prescription process from the time the patients visits the doctor, to the time the patient receives the medication and the pharmacy is being reimbursed from the insurance fund, has been analyzed in its full complexity. The new electronic system has been developed taking into consideration all these requirements and prerequisites. The new system has been able to overall improve the process of drug prescribing from the safety perspective (less medication errors), level of service (more interpersonal communication and less administrative overload with the patient), economical aspect (mainly as far as the public expenditure is concerned) and back office administration. As any other new system, it has been also identified that most users struggle to familiarize, spend more time especially at the beginning while trying to learn how to use it. Definitely, there is room for improvement for the system itself but mainly for the organized, well-structured and extensive training of the users. Pharmacists find EPS easier and faster to process an e-prescription, feel safer regarding medication and dispensing errors and are able to provide more time for advising and communicating with the patients. Patients feel safer from potential medication errors, are better satisfied with the level of service and the time spent with their pharmacists and believe the whole system in financially more efficient and saves their insurance money. Doctors, despite struggling with the new system's various complexity, feel much safer that their patients will receive the correct medication at the right price. All of the groups agree that the EPS system is a major step towards medication safety, can modernize and eventually save the health professional some significant time which will then result in improved interpersonal communication of the patient with his/her doctor or pharmacist. The EPS has significantly rationalized the total healthcare costs and unnecessary expenditure and there is room for improvement regarding the training and readiness of the health professionals to better use the new system.

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# SAFETY OF OTC ANALGESIC DRUGS IN THE OPINION OF POLISH PATIENTS - PRELIMINARY STUDY

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**Abstract:** The aim of the paper was to determine the Polish patients' attitude towards self-medication with OTC products. The research was based on a questionnaire for adult patients. In all, 363 questionnaires were collected between February-April 2011. There were used three data collection methods. Direct interviews were performed by an interviewer. Additionally, respondents provided answers to the questionnaire in a self-administered way and questionnaires were filled by the Internet users. The collected data were analyzed in the MS Excel program. Women comprise a group using more frequently analgesics and medicines against common cold; however, men present more symptoms caused by an improper use of these drugs. People over 60 years mostly use simultaneously products having the same active compound, thinking that OTC products are safe. Respondents' education is the major differentiating factor. People with primary and secondary education usually combine 2-3 products during common cold treatment and in their opinion OTC drugs are safe and difficult to overdose. Respondents with high school education more frequently use the drugs according to their own choice and abuse them in case of symptoms exacerbation. In the article, Polish patients' attitude towards the use of self-medication products available without prescription has been presented. The respondents use analgesics and products against common cold in a way, which significantly differs from the recommendations. The reports about overdosing, misuse of medicines and polypharmacy phenomenon should induce more intensive efforts of pharmacists in providing advice to patients who are using OTC products in treating themselves.

**Keywords:** over the counter drugs, pharmacovigilance, self-medication, analgesic drugs, opinion of patients

The main objective of health care system is the health safety of the general population. There are two main objectives and functions of the system identified at the same time. One of them is to meet individual health needs resulting from illness or accidents, and the second one is to ensure the collective health care needs including, for example, living conditions, employment, housing, nutrition, rest. Pharmacotherapy occupies an important place in the process of health care and the parameter that characterizes the value of the drug, in addition to its effectiveness is its safety profile (1).

Self-medication is a vital part of the health care system, which has dynamically evolved in recent years. However, in Poland, patients' knowledge about the health and the use of drugs is rather low, compared to Western Europe. This is due to insufficient access to reliable information about medicines. Moreover, in contrast to, for example, the United Kingdom, the policy of the Polish government is still not focused on promoting self-medication. Therefore, its development is not proceeding in Poland as a natural process, but it is due to lack of adequate access to medical care, and therefore often regarded as a necessary evil (2, 3).

Self-use of various therapies involves benefits and risks. The majority of Polish people try to solve their health problems themselves. Patients have increasing access to drugs, but despite the increased availability of medicines outside pharmacies, it is the pharmacist and the doctor who should verify the safety of medicines usage.

An important element of self-medication is the use of non-prescription medicines. Modern drugs belonging to the OTC (over the counter) drugs or medicines accepted to trading without a prescription, are widely available and can be found in almost any grocery store or gas station. From the patient's

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perspective, these changes are very beneficial, because the access to OTC products becomes unlimited. However, from the point of view of pharmacology safety, a wide and unlimited access to medicines is the cause of some health problems (4).

When using OTC drugs, patients can prevent minor disorders, cure them, and thereby improve their quality of life. Patients are no longer passive spectators controlled by health care entities, but are actively involved in the treatment process and become partners for a physician and a pharmacist. They take the responsibility in the process of their treatment and, based on their own or the expert opinion, they have the possibility to decide on the drugs to be used. The process of self-medication is also cost-effective for the National Health Fund (NHF), because apart from reducing the cost of pharmacotherapy reimbursed by shifting its financing, patients can avoid unnecessary visits to the doctor. Prophylactic drugs that reduce the risk of disease and even hospitalizations also have rated benefits (2).

It is therefore important to introduce pharmaceutical care, which, due to widely carried patient education and advising, could significantly limit the risk that accompanies the acquisition of OTC medicines outside pharmacies. Grocery store, hypermarket and gas station do not employ qualified persons. A pharmacist is the person who can instruct how to take the medicine, not to mention its right choice. Outside pharmacy, patients themselves make decisions based on what they or their family know about medicines and pharmacotherapy (5).

Another equally important part of pharmaceutical care is to monitor the safety of pharmacotherapy. This obligation is regulated by the Act of the Pharmaceutical Law where the Article 88 lists that one of the responsibilities of the pharmacy is to provide the President of the Office for Registration of Medicinal Products with adverse event information about a medicinal product or a medical device (ADR) (6). In addition, The Health Ministry Order dated 17 February 2003 on the drug safety monitoring, paragraph 9.4, requires that doctors, pharmacists and other health professionals report adverse reactions of medicinal products. Such a legal regulation does not exist in relation to institutional staff for sales out of pharmacies, however, the European Parliament and the Council 2010/84/EU has given the patients the right to direct reporting of adverse drug reactions.

In the era of transition, when an increasing number of drugs are changing the status from prescription only medicines (POM) (Rx) to OTC, the obligation to report data on adverse reactions lies increasingly with the pharmacist. Communication of information to patients about the side effects of OTC drugs and interactions between the drugs, also with those issued on prescription, is particularly important because it raises the standards of health care (7). The studies performed in the European Union and the United States have shown that the side effects of self-medication are one of the leading causes of deaths, with about half of them which could have been avoided (8, 9). The fact that the drug has been registered does not mean that it has been tested in every possible way. Definitely, it had to undergo many tests and meet certain strict standards, but the knowledge about all possible interactions and side effects (sometimes, also those very distant) is possible to be collected only during the post-registration phase of research (7).

Patients using non-prescription medicines believe that if the products can be bought without a prescription, they are safe and do not pose a risk in relation to their use. Even the vitamins can cause damage if taken in excess. The fact that some of the analgesics are used in committing suicides should raise awareness on how dangerous they can be, e.g., paracetamol is in the UK the most common cause of suicidal drug poisoning (7, 10). Due to adverse reactions after ingestion of drugs in the group of non-steroidal anti-inflammatory drugs (NSAIDs), 30 thousand of hospitalizations are recorded annually. After using NSAIDs, 1.2-2 thousand of deaths per year are noted in relation with adverse drug reactions (approximately the same size of deaths as due to AIDS). In Poland, the estimated number of deaths due to NSAIDs caused adverse reactions is 200-3000 deaths per year (11). These data indicate the magnitude of the problem we are facing. In Poland, the most commonly used medicines available at pharmacies without prescription are the analgesics. It is estimated that about 30% of the older patients' population are taking these drugs habitually (12).

On the pharmaceutical market, there is currently a large selection of analgesics available. Among them, the most important are the NSAIDs, especially paracetamol, ibuprofen and metamizole sodium. They are used principally to control headache, muscular pain, dental pain, menstrual disorders, or to reduce fever. Advertisements in the mass media present them as drugs which are safer, smarter, and that ideally appeal to pain location. In principle, their use should not cause major problems, but the reality is different. Bleeding from the gastrointestinal tract, liver damage, kidney failure, are some of the side effects that may occur quite frequently (13,

14). After using NSAIDs, rarely some serious adverse reactions in the skin can be observed, such as maculopapular rash, Stevens-Johnson syndrome (bullous erythema multiforme), toxic epidermal necrolysis, cutaneous hypersensitivity reactions including skin rash (15). The occurrence of these side effects is not always associated with an OTC NSAIDs drug usage.

Risk in relation with the use of medicines, especially the NSAID group, appears when patients very commonly use simultaneously a combination of several drugs from the same group, which increases the risk of adverse reactions, rather than strengthening the therapeutic effect. In order to improve safety of the pharmacotherapy and to minimize adverse drug reactions, the methods for a proper drugs use should be carefully analyzed. The expertise of doctors and pharmacists is indispensable since patients are not able to solve these problems by themselves. Appropriate use of medicines and medication reduce the risk of pharmacotherapy errors committed by both patients and health care professionals.

**Objective**

The aim of this study was to evaluate patients' perception of the safety of analgesics available without prescription.

**Methods**

The research material comprised data collected on the basis of an original questionnaire addressed to adults. This questionnaire was validated in a group of 50 people. Overall, there were analyzed 364 questionnaires completed by the interviewer in a direct interview with the patient or individually by the respondent. The survey was conducted between February and April 2011, mainly among the patients in outpatient clinics, as well as among the Internet users at the social networking site "Facebook" and www.insomnia.pl forum. The data obtained were statistically analyzed using the MS Excel 2007.

**RESULTS**

The study presents the results collected in a heterogeneous group, i.e., with a different way of access to the respondents. In this study, it can be assumed that only the age of the respondents could have an impact on this method, affecting the answers to the other questions in the survey related to the perception of analgesics. Therefore, it should be considered that the study relates more to the young Polish population. The data collection methods did not affect the remaining features like gender, education and place of residence.

**Demography**

The results of demographic characteristics of respondents are shown in Table 1.

As shown in the presented data, the thesis placed in the introduction part of the results was confirmed. The respondents are mainly young people, which indicates the calculated median of 29 years.

**Use of OTC drugs**

In this research, it was decided to ask the respondents about the frequency of using analgesics acquired without a medical prescription. The results are shown in Table 2.

In the interview, we found that among the respondents who most frequently used the analgesics, the medicines containing paracetamol were mainly preferred. Their application was not always consistent with the indications. For example, one of the patients for over a year had taken "Apap night" every single day as a hypnotic drug. It is a formulation containing paracetamol and diphenhydramine used in the short-term pain causing difficulty in falling asleep. The use of this drug can cause com-

Table 1. Characteristics of respondents (n = 364 persons).

| | |
|---|---|
| Age (year) | min = 18 max = 85<br>average = 36.3 median = 29 SD = 15.5 |
| Sex | female = 228 male = 136 |
| Degree | education primary = 21<br>education secondary = 139<br>education higher – incomplete = 66<br>education higher = 138 |
| Abode | city over 100 thousand inhabitants = 185<br>city under 100 thousand inhabitants = 80<br>country = 99 |

Table 2. Frequency of use of analgesics OTC.

| | Every day n (%) | Few times a week n (%) | Once a week n (%) | Few times a month n (%) | Once a month n (%) | Few times a year n (%) | I do not use n (%) | $\chi^2$ | df | p |
|---|---|---|---|---|---|---|---|---|---|---|
| Total (n = 364) (27) | 10 (2.7) | 23 (6.3) | 9 (2.5) | 94 (25.8) | 64 (17.6) | 135 (37.1) | 29 (8.0) | - | - | - |
| Female (n = 228) | 8 (3.5) | 17 (7.5) | 9 (3.9) | 66 (28.9) | 47 (20.6) | 72 (31.6) | 9 (3.9) | | | |
| Male (n = 136) | 2 (1.5) | 6 (4.4) | 0 (0) | 28 (20.6) | 17 (12.5) | 63 (46.3) | 20 (14.7) | 30.8 | 6 | < 0.0001 |
| Primary education (n = 21) | 1 (4.8) | 2 (9.5) | 0 (0) | 7 (33.3) | 2 (9.5) | 9(42.9) | 0 (0) | | | |
| Secondary education (n = 139) | 8 (5.8) | 11 (7.9) | 5 (3.6) | 35 (25.2) | 23 (16.5) | 43 (30.9) | 14 (10.1) | | | |
| Incomplete higher education (n = 66) | 0 (0) | 2 (3) | 1 (1.5) | 17 (25.8) | 13 (19.7) | 25 (37.9) | 8 (12.1) | | | |
| Higher education (n = 138) | 1 (0.7) | 8 (5.8) | 3 (2.2) | 35 (25.4) | 26 (18.8) | 58 (42) | 7 (5.1) | 21.9 | 18 | 0.238 |
| City over 100,000 inhabitants (n = 185) | 2 (1.1) | 6 (3.2) | 5 (2.7) | 44 (23.5) | 32 (17.3) | 77 (41.6) | 19 (10.3) | | | |
| City under 100,000 inhabitants (n = 80) | 1 (1.3) | 3 (3.8) | 4 (5) | 26 (20) | 16 (20) | 26 (32.5) | 4 (5) | | | |
| Country (n = 99) | 7 (7.1) | 14 (14.1) | 0 (0) | 24 (24.2) | 16 (16.2) | 32 (32.3) | 6 (6.1) | 33.8 | 12 | 0.001 |
| Age 18-30 (n = 190) | 0 (0) | 5 (2.6) | 1 (0.5) | 44 (23.2) | 47 (24.7) | 77 (40.5) | 16 (8.4) | 71.4 | 18 | < 0.0001 |
| Age 31-45 (n = 66) | 1 (1.5) | 4 (6.1) | 3 (4.5) | 24 (36.4) | 6 (9.1) | 24 (36.4) | 4 (6.1) | | | |
| Age 46-60 (n = 71) | 3 (4.2) | 8 (11.3) | 4 (5.6) | 18 (25.4) | 11 (15.5) | 23 (32.4) | 4 (5.6) | | | |
| Age over 61 (n = 38) | 6 (15.8) | 6 (15.8) | 1 (2.6) | 9 (23.7) | 0 (0) | 11 (28.9) | 5 (13.2) | | | |

mon side effect in the form of somnolence, disturbance in attention, dizziness, dryness of mucous membranes in the mouth, throat and nose and rare hypersensitivity reactions i.e., rash, hives, swelling and angioedema.

The statistical analysis has shown that at the level of statistical significance $\alpha = 0.05$ you cannot reject the hypothesis that the tested variables are independent of sex, age and place of residence. On the other hand, there is no reason to reject the null hypothesis that the tested variables are independent of the education of the respondents.

In this study, the respondents were asked about the frequency of using OTC drugs containing NSAIDs in case of common cold . The results are shown in Table 3.

The statistical analysis results as regards to gender, education and place of residence have demonstrated that the tested variables are on the border of statistical significance. Assuming the statistical significance at the level of $\alpha = 0.05$, the null hypothesis that the tested variables are independent of the studied ranges must be rejected.

Subsequently, the respondents were asked about the use of OTC drugs containing paracetamol in their composition, taken during a common cold. The results are shown in Table 4.

The statistical analysis has shown that at the level of statistical significance $\alpha = 0.05$ there is no reason to reject the null hypothesis that the tested variables are independent of the gender, age and place of residence. On the other hand, the hypothesis that the tested variables are independent of the education of respondents must be rejected.

In the next phase of the research, the respondents answered the question on the use of medicines containing paracetamol to treat headaches caused by alcohol consumption. The results are shown in Figure 1.

The statistical analysis of the results shown in the graph has confirmed that studied variables at significance level $\alpha = 0.05$ are dependent on gender, education and age, but there is no reason to reject the null hypothesis that the tested variables are independent of the place of residence.

**Risk of adverse reactions after OTC medicines use assessed by patients**

In the following research phase, the patients were interviewed about the safety and risks associ-

Table 3. Frequency of use of OTC drugs during a cold composed containing NSAIDs

| | In exceptional cases: severe pain, high fever n (%) | Every time I am ill n (%) | I do not use n (%) | $\chi^2$ | df | p |
|---|---|---|---|---|---|---|
| Total (n = 364) | 199 (54.7) | 159 (43.7) | 6 (1.6) | - | - | - |
| Female (n = 228) | 117 (51.3) | 109 (47.8) | 2 (0.9) | 5.8 | 2 | 0.054 |
| Male (n = 136) | 82 (60.3) | 50 (36.8) | 4 (2.9) | | | |
| Primary education (n = 21) | 8 (38.1) | 11 (52.4) | 2 (9.5) | 12.6 | 6 | 0.051 |
| Secondary education (n = 139) | 82 (59) | 56 (40.3) | 1 (0.7) | | | |
| Higher education – incomplete (n = 66) | 35 (53) | 29 (43.9) | 2 (3) | | | |
| Higher education (n = 138) | 74 (53.6) | 63 (45.7) | 1 (0.7) | | | |
| City over 100,000 inhabitants (n = 185) | 110 (59.5) | 71 (38.3) | 4 (2.2) | 8.3 | 4 | 0.081 |
| City under 100,000 inhabitants (n = 80) | 35 (43.8) | 45 (56.2) | 0 (0) | | | |
| Country (n = 99) | 54 (54.5) | 43 (43.4) | 2 (2.1) | | | |
| Age 18-30 (n = 190) | 102 (53.7) | 85 (44.7) | 3 (1.6) | 7.3 | 6 | 0.291 |
| Age 31-45 (n = 66) | 38 (57.6) | 25 (37.9) | 3 (4.5) | | | |
| Age 46-60 (n = 71) | 42 (59.2) | 29 (40.8) | 0 (0) | | | |
| Age over 61 (n = 37) | 18 (47.4) | 20 (52.6) | 0 (0) | | | |

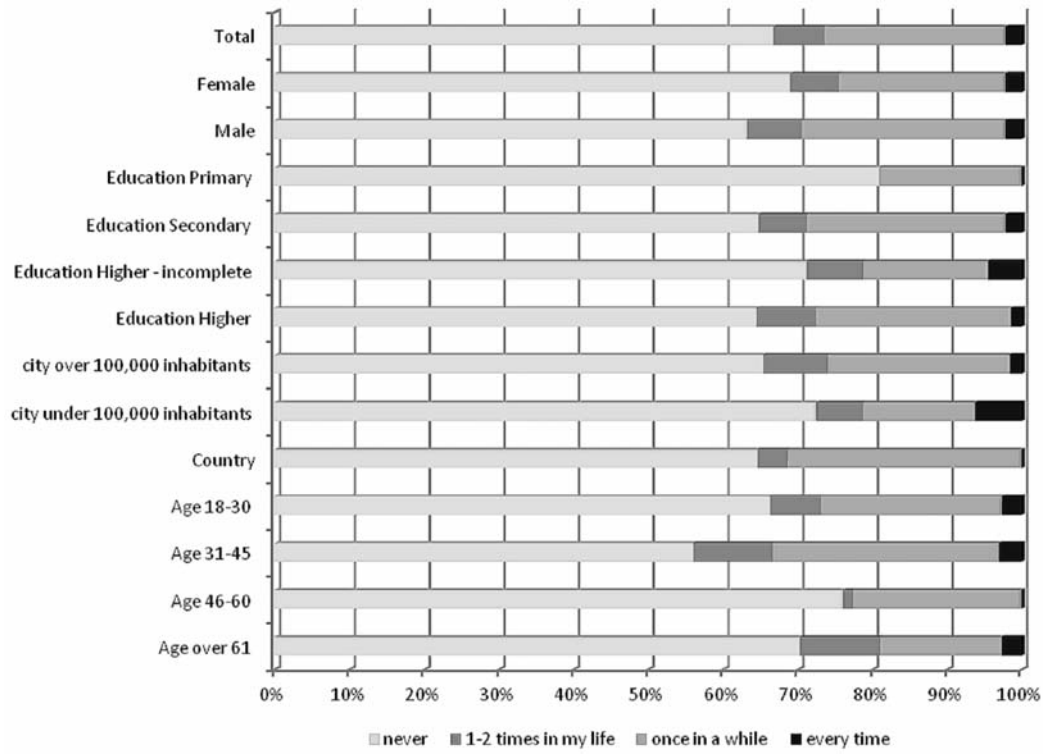


Figure 1. Frequency of analgesics' containing paracetamol use as a treatment for headaches caused by alcohol (Statistical analysis: gender: $\chi^2 = 1.37$; df = 3; p = 0.714; education: $\chi^2 = 7.6$; df = 9; p = 0.577; place of residence: $\chi^2 = 43.3$; df = 6; p < 0.00001; age: $\chi^2 = 11.4$; df = 9; p = 0.247)

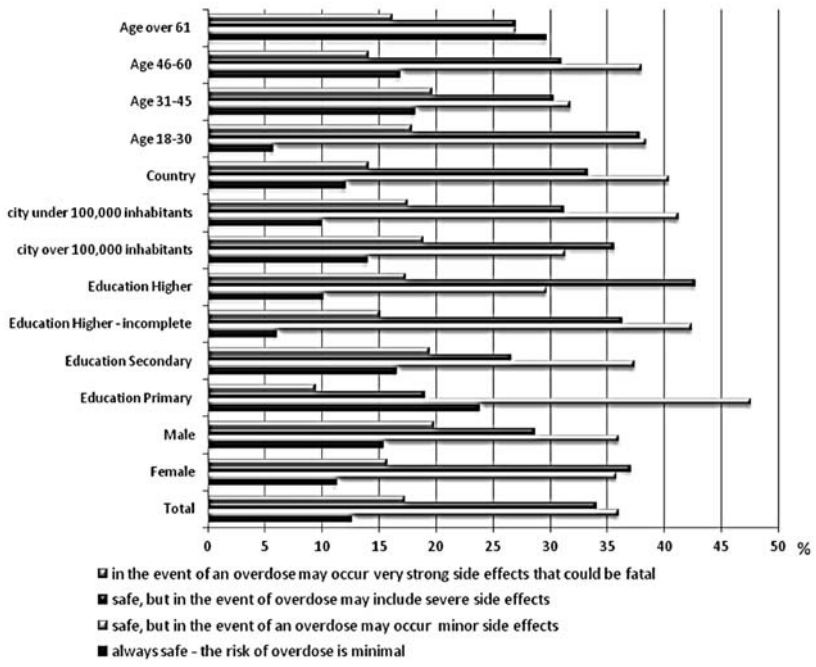


Figure 2. The risk assessment of the analgesics' use and medications against common cold containing paracetamol (Statistical analysis: gender: $\chi^2 = 4.0$; df = 3; p = 0.26; education: $\chi^2 = 17.9$; df = 9; p = 0.036; place of residence: $\chi^2 = 4.2$; df = 6; p = 0.649; age: $\chi^2 = 22.9$; df = 9; p = 0.007)

ated with the very common self-medication by the patients. The results are summarized in Figure 2.

The statistical analysis of the results shown in the graph has demonstrated that the studied variables at significance level $\alpha = 0.05$ are dependent on gender and place of residence, but there is no reason to reject the null hypothesis that the tested variables are independent of the education and age of the respondents.

## DISCUSSION

Self-medication is one of the elements of the health care system important for both the patient and the State. Continuous increase of patients' access to medicines results in reduction of the burden on doctors' visits in relation to symptoms that patients assess as minor and where they themselves are able to diagnose. Therefore, self-medication is one of the potentials for saving public finances and at the same time, allowing a conscious patients responsibility for their own health (16).

Among the respondents more than 54% of the interviewed said that they used drugs during treatment only in exceptional cases, such as severe pain and high fever treatment, and nearly 44% use them every time they get sick, regardless of the severity of symptoms. Less than 2% of respondents reported that they did not use drugs during a common cold. It is worth mentioning that the use of antipyretics may not always be beneficial. It is necessary to make patients aware that high temperature, which is accompanied by a viral infection, is only a defensive reaction of the organism and it should not be lowered every time, e.g., temperature 39°C induces the production of interferon, which shortens the course of the disease (17). Only in case of small children, in persons with lowered threshold for seizures, and with cardiovascular diseases, fever increase should be avoided as it may compromise the health or even patient's life (18).

Respondents when asked about the use of compound preparations containing paracetamol answered in nearly 62% that in case they used a combined preparation it was always the same choice. Twenty-eight percent of patients combined two products, and 3% indicated that they combined even three products. However, 7% of respondents did not use complex formulas. It should be emphasized that a combination of two products containing paracetamol is wrong, since it can result in exceeding the threshold for paracetamol dose (1000

Table 4. Usage of common cold preparations with paracetamol.

| | I do not use n (%) | Always uses only one particular drug n (%) | Sometimes I combine the two drugs n (%) | Sometimes I combine the three drugs n (%) | $\chi^2$ | df | p |
|---|---|---|---|---|---|---|---|
| Total (n = 364) | 27 (7.4) | 223 (61.3) | 102 (28) | 12 (3.3) | - | - | - |
| Female (n = 228) | 11 (4.8) | 150 (65.8) | 61 (26.8) | 6 (2.6) | 8.7 | 3 | 0.033 |
| Male (n = 136) | 16 (11.8) | 73 (53.7) | 41 (30.1) | 6 (4.4) | | | |
| Primary education (n = 21) | 2 (9.5) | 9 (42.9) | 8 (38.1) | 2 (9.5) | 9.7 | 9 | 0.378 |
| Secondary education (n = 139) | 7 (5) | 87 (62.6) | 41 (29.5) | 4 (2.9) | | | |
| Higher education – incomplete (n = 66) | 6 (9.1) | 36 (54.5) | 21 (31.8) | 3 (4.5) | | | |
| Higher education (n = 138) | 12 (8.7) | 91 (65.9) | 32 (23.3) | 3 (2.2) | | | |
| City over 100,000 inhabitants (n = 185) | 17 (9.2) | 120 (64.9) | 43 (23.2) | 5 (2.7) | 10.6 | 6 | 0.100 |
| City under 100,000 inhabitants (n = 80) | 5 (6.3) | 51 (63.8) | 20 (25) | 4 (5) | | | |
| Country (n = 99) | 5 (5.1) | 52 (52.2) | 39 (39.4) | 3 (3) | | | |
| Age 18-30 (n = 190) | 10 (5.3) | 113 (59.5) | 62 (32.6) | 5 (2.6) | 16.7 | 9 | 0.053 |
| Age 31-45 (n = 66) | 9 (13.6) | 46 (69.7) | 10 (15.2) | 1 (1.5) | | | |
| Age 46-60 (n = 71) | 6 (8.5) | 44 (62) | 17 (23.9) | 4 (5.6) | | | |
| Age over 61 (n = 38) | 2 (5.3) | 20 (52.6) | 13 (34.2) | 3 (7.9) | | | |

mg/day) and will intensify the effects of drug-induced toxicity without increasing efficacy (19). Complex medicinal products, used against common cold containing in their composition paracetamol and available on the Polish pharmaceutical market include 250 to 1000 mg of pure substances in a single dose (17). In a study performed at the Department of Dermatology of Military Institute of Medicine in Warsaw, the costs of dermatologic drug reactions were assessed. Among others, it was found that in the case of dermatological adverse reactions as erythema multiforme, which could be an adverse reaction after using paracetamol, the generated cost of the adverse event treatment ranged from 2853.03 PLN to 5277.54 PLN. In case of other drug reaction, like toxic-allergic dermatitis, the treatment cost generated was at the level ranging from 2814.87 PLN to 3003.87 PLN (20).

In the case of NSAIDs, caution should be kept. Even one taken tablet may be harmful if patient suffers from peptic ulcer disease, renal or cardiovascular disease. The problem may concern also potentially healthy patients, unaware of the risk related to treatment with the medicines as recalled by J. Woroń (21). Patients should not combine NSAIDs with alcohol as this combination represents some risk (22) and in some cases, even a single use can cause serious health problems (23). In response to a question about the use of medicines containing paracetamol to treat headaches caused by alcohol consumption, only 2.2% of the interviewed answered that they used it every time, 24.2% of respondents did this from time to time and 6.9% so far took these drugs only 1-2 times in their life. Among the respondents, 66.9% of them denied taking paracetamol after drinking alcohol.

When analyzing the results with respect to gender, it has been found that women often use the medications for common cold. The percentage of interviewed subjects using these products whenever they become ill is 47.8% and in exceptional cases, such as severe pain or high fever – 51.5%. Only 0.9% of women do not use any OTC drugs during a common cold. Men often responded that they used products to treat common cold only in exceptional cases – 60.3%, and each time they had common cold symptoms they were using them in only 36.8%, while 2.9% declared not using any drugs. Both women (2.6%) and men (4.4%) rarely combine three preparations containing paracetamol. More often, they use two products at the same time, men 30.1% and women 26.8%. However, a significant differences are marked by the use of those medications in general, 11.8% of men stated that during the common cold did not use any drug containing paracetamol, and in case of women, the figure is 4.8%. Men compared with women are slightly more often using drugs for headaches caused by alcohol consumption. This is because men consume more frequently and bigger amounts of alcohol (24) and become addicted to alcohol 2.5 times more likely than women (25). Among men, the answer that the product is used from time to time was positive in case of 27.2% of the respondents, among women it was 22.3%. People who use products fighting headache every time they experience headache caused by alcohol are 2.2% in both sexes.

Based on the results of the research it was found that most frequently medications for common cold treatment were used by elderly patients: 52.6% of people over 60 years take the medicines each time in the event of a cold, while 47.4% of this population use the medicines only in exceptional cases. Rarely products for common cold treatment are used by those aged 31-45 years. The usage of medicines each time common cold affects them is declared by 37.9% of people, while 4.5% of people do not use any drugs in the event of illness. It can be observed that the group of people who most frequently combine three formulations of paracetamol comprises people over the age of 60 years (7.9%) and aged 46-60 years (5.6% of respondents). The lowest percentage of people using a combination of three formulations is in the range of 30-45 years (1.5%). Concomitant treatment with two or more NSAIDs can lead to their competition for binding and inhibition of the COX enzymes and altered time course of the pharmacological effects (26). The respondents aged 30-45 years who did not use any products containing paracetamol constituted 13.6%. In the group of respondents over 60 years, 34.2% of patients are using simultaneously two products, and this is the highest percentage in the group of respondents, while 5.6% of them declare using even three preparations at the same time. Also in this age group there was reported the least number of respondents who always used only one medication (52.6% of respondents). The analysis of responses by age groups indicated that the group that most often used medicines for headaches as pain relief drugs after drinking alcohol, were young people. Taking medication from time to time in the above-mentioned indication was declared by 30.3% of respondents in the age range of 31-45 years. Slightly less, 24.2% of these people are aged between 18-30 years. The percentage of respondents, who never used medication for that purpose, is as follows: 18-30 years – 66.3%, 31-45 years – 56.1%. The proportion of those who

declared that it never happened to them to take medicines in case of headache after alcohol consumption is the highest in the 45-60 years interval and it is 76.1%; however, looking at those who declared using the medication for that purpose from time to time, it is still applicable to 22.5% of respondents. Respondents over 60 years of age rarely use drugs with paracetamol for a headache caused by alcohol consumption, although studies show that an older age (> 65 years) male abuse of alcohol is about four times more often than women. The prevalence of alcohol abuse decreases with age and for the elderly it is about 3% (24). Among them there is a very high percentage of people who have never used such preparations for the mentioned above purpose (70.3%). The answer that they use it from time to time was positive only in 16.2% of respondents, and the answer that they used it 1-2 times in their whole life was 10.8%. The percentage of people who use it every time after drinking alcohol is fixed at 2.7%. The exceptions are those aged 45-60 years as none of the people here did answer "every time."

Analyzing the results by the level of respondents' education, those with primary education often use drugs for common cold treatment (52.4%), however, within this group, there is also the largest number of people (9.5%) not using any medication in case of illness. In the other groups, no significant differences have been observed. People with primary and secondary education, often combine several drugs containing paracetamol. In the primary education group, the percentage of people who used only one product is 42.9%, while 38.1% of people use a combination of two different products containing paracetamol. In this group, the percentage of respondents declaring the use of three products at the same time is the highest, and it is 9.5%. The respondents with secondary education constitute the largest group using only one medication (62.6%). In the group of respondents with higher education and in the higher incomplete education group, half of them used one (high education incomplete – 54.5%, university degree – 65.9%) or two preparations (high incomplete – 31.8%, university degree – 23.2%). The proportion of respondents who declared using a total of three preparations is 4.5% among those with incomplete high education, and only 2.2% in the group with university degree.

We found that the persons with primary education take most rarely analgesics for the headache after alcohol intake. No respondents in this group reported using them "every time" and 19% do it "from time to time". However, the answer that "it never happened" was in case of 81% respondents, the highest score in this patient population. The group, which most frequently used products with paracetamol, includes people with secondary and university education, the percentage of people who use it "from time to time" is 26.1-26.6%. However, the largest proportion of respondents who use them "every time" is among those with incomplete university education (4.5%). When analyzing this information, it was demonstrated that regardless of the place of residence the highest percentage of respondents used NSAIDs without medical prescription few times a year or several times a month. Almost one third of people living in rural areas indicated the answer "from time to time", none of them stated that "every time". The respondents living in rural areas declared that they never used NSAIDs (64.6%). The highest percentage of people not using NSAIDs is among the urban population up to 100 thousand. To the question about the use of OTC medicines for common cold, 59.5% of people living in large cities and 54.4% of people living in rural areas said that they used them in case of high fever or pain. However, 56.2% of residents of smaller cities use them whenever they suffer from a common cold, and it is the highest percentage of respondents. When analyzing the answers regarding combining products containing NSAIDs, it was found that most respondents used one particular formulation. Only those living in the countryside almost double the percentage of using two products at the same time than urban residents. Among respondents taking medicines containing paracetamol for pain associated with alcohol consumption, most people, regardless of their place of residence, answered that they "do not use it". However, "from time to time" the medicines are used by 31.3% of rural residents, while none of this group answered using them every time.

Later in the study, there was an attempt to assess patients' knowledge about the safety of OTC use. Safety depends also on the place of drug acquisition. The place most frequently mentioned by patients where NSAIDs were purchased is a pharmacy (27). In the assessment of the safety of OTC drugs, the interviewed responded very differently. Most of them stated that in their opinion, the OTC products were safe, however detailed opinions about the possible side effects were different. A possibility of observing minor side effects during treatment was confirmed by 36% of respondents, while 34.1% chose the answer that "severe side effects" could happen. Among the respondents, 17.3% said that an overdose can lead to death of the patient and 12.6% believed that these drugs were always safe and the

risk of overdosing is minimal. During the interview, respondents very often stressed that it was a very difficult question for them to answer, because they did not have sufficient knowledge to be able to assess the risks caused by an overdose of analgesics and of products used for the common cold treatment. It should be noted that patients using pharmacist's services (purchased from a pharmacy) had the opportunity to get complete and reliable information on the drug, which they wanted to acquire. This is one of the important aspects of pharmacist's profession, to provide advice in the process of self-medication (28).

The analysis has demonstrated that the analgesics cause the highest concern among young people. In the group of respondents aged 18-30 years, 17.9% of people defined analgesics as very dangerous in case of overdosing. These results can be explained by the fact that young people often use the Internet and other sources of information, choose by themselves the medication and treatment methods. Perhaps this is the reason of their greater knowledge about the medicines than among the elderly. Older people often rely on the expertise of pharmacists and physicians, as mentioned by the respondents while completing the questionnaire (27). However, only 5.8% of them think that the analgesics are safe, and the risk of overdose is minimal. Very similar results regarding the possibility of experiencing adverse drug reaction were obtained from respondents aged 31-60 years and in the youngest group (18-30 years), but the opinion about safety and the impossibility of overdosing was three times higher. The biggest differences regarding the answers related to safety, possible overdose and adverse effects were in the group of respondents over 60 years of age. In these patients' group 16.2% declared that in their opinion these drugs could be life threatening, and 29.7% considered them to be very safe. The obtained result is quite worrying, as according to previous studies, this is the group using analgesics most commonly and in the largest quantities (29, 30). Such observation is also confirmed by this research – those over 60 years of age combine several NSAIDs in higher percentage than the younger people.

In the assessment of OTC drugs safety also an analysis according to patients' education level was performed. The analysis indicates that the higher the level of education of respondents, the higher risk awareness about the use and abuse of analgesics and drugs against common cold. People with primary education have the greatest confidence towards medicines as 23.8% believe that the NSAIDs are completely safe and the risk of overdose is minimal, minor side effects are expected by 47.6% of the respondents in this group of patients. On the other hand, 19% think that an overdose can give strong side effects, but only 9.5% said that its abuse could be fatal. In comparison, within the group of patients with incomplete university education the percentage of those who think that medicines are safe is only 6.1%. Among people with university education it corresponds to 10.1%, however, the vast majority of respondents within this group have chosen the answer confirming that there is risk awareness, 42.8% believe that there may be severe side effects, and 17.4% believe that an overdose can be fatal. The most diverse in terms of response was a group with high school education: 16.5% of respondents considered the NSAIDs to be always safe and 19.4% - as unsafe and potentially life threatening in case of an overdose. Minor side effects are expected by 37.7% of them, and the severe – by 26.6%. It is worrying that regardless of the place of residence for almost 40% of people the use of OTC drugs is safe, in case of overdosing there may be slight side effects. In addition, for 10% of respondents living in small towns and over 14% of those living in large cities, the studied OTC drugs are considered to be always safe and according to them, the risk of overdosing is minimal.

## CONCLUSION

This paper presents the importance of self-medication and attitude of Polish people towards drugs available without a prescription in the Polish health care system. It can be easily observed that the way in which respondents use analgesics and drugs against cold differs significantly from the recommendations, even if they are included in the leaflets. Polish people during the process of self-medication typically use medical products only in exceptional cases, and to a lesser extent, each time whenever they become sick. Generally, they use paracetamol in complex preparations of their choice. Quite often, they combine two or even three of such preparations. The vast majority denies receiving paracetamol after alcohol consumption, although it is clear that a single drug abuse or combining the medicine with alcohol does not significantly affect the health of the patient. However, if done frequently, it can cause some unexpected side effects.

Patients believe that OTC NSAIDs are safe, although opinion on the possibility of adverse effects indicates them not to be fully safe. These drugs arouse the biggest concern among the young people. Therefore, reports of overdose, abuse and

polypharmacy phenomenon should lead to the adoption of intensive work of a pharmacist in providing information to patients who use the OTC products in self-medication process.

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## SHORT COMMUNICATION

# NEUROPHARMACOLOGICAL AND TOXICITY STUDY OF NEWLY PREPARED *N*-[5-(3-CHLORO-4-FLUOROPHENYL)-1,3,4-THIADIAZOL-2-YL]-2-SUBSTITUTED ACETAMIDES

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**Abstract:** Various *N*-(5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl)-2-substituted acetamides were synthesized starting from 3-chloro-4-fluorobenzaldehyde. Structures of the compounds were confirmed on the basis of spectral data. The compounds were evaluated for their anticonvulsant activity. Neurotoxicity and hepatotoxicity studies were also performed. Interestingly, out of ten compounds, three compounds (**VIb, VIe** and **VIg**) were found to be effective at a dose of 30 mg/kg. Compounds **VIb** and **VIe** showed slight neurotoxicity whereas **VIg** was found to be free from any neural impairment. Out of the six compounds examined for hepatotoxicity only compound **VIc** showed an elevated alkaline phosphatase value with $p < 0.01$.

**Keywords:** acetamide, acetazolamide, anticonvulsant activity, neurotoxicity, hepatoxicity

Epilepsy is a central nervous system (CNS) disorder characterized by paroxysmal cerebral dysrythmia, manifesting itself as brief episodes (seizures) of loss or disturbances of consciousness, with or without characteristic body movements (convulsions), sensory or psychiatric phenomena (1). Approximately 1-2% people in all nations and of all races are affected by epileptic disorder (2, 3). The prevalence of epilepsy and other seizure disorders in Saudi population were estimated approximately 0.65% (4). Over 30% epileptics do not have seizure control even with the best available medications (5).

Thus, the search for newer antiepileptic agents with more selectivity and lower toxicity continues to be an area of investigation in medicinal chemistry (6). In the effort to get the potent antiepileptic agents, several five and six membered heterocyclic compounds were synthesized and had shown considerable anticonvulsant activities (7, 8).

Acetazolamide and methazolamide are well known antiepileptic drugs with 1,3,4-thiadiazole moiety incorporated into acetamide group. Moreover, literature sources have proven that other derivatives of 1,3,4-thiadiazole are also potent anticonvulsant agents (9, 10). Attaching substituted acetamides with 1,3,4-thiadiazole is expected to increase the antiepileptic activity by producing the synergistic effect. Many antiepileptic drugs have been discovered on the basis of structural similarities (11).

The structural similarities between title compounds and acetazolamide (or methazolamide) along with some recently reported antiepileptic agents (12-14) prompted me to design and synthesize several *N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-substituted acetamides for antiepileptic screenings. Interestingly, these compounds were found to have appreciable anticonvulsant activity with lower neuro- and hepatotoxicity.

## EXPERIMENTAL

### Chemistry

Melting points were taken in open capillary tubes and are uncorrected. $^1$H-NMR spectra were recorded on a Bruker model DRX 300 NMR spectrometer in $CDCl_3$ using tetramethylsilane (TMS) as an internal standard. IR spectra were recorded on BIO-RAD FTS 135 spectrometer using KBr pellets. TLC was carried out using silica gel G as stationary phase and toluene : ethyl acetate : formic acid (5 : 4 : 1, v/v/v) as mobile phase for all the obtained compounds. All the chemicals and solvents used were obtained from Merck. All kits used in the present study were the products of Biodiagnostic Co.

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(Egypt), Biosystems Co. (Spain) and Randox Laboratories (U. K.).

**Synthesis of (*E*)-*N*-(3-chloro-4-fluorobenzylidene) hydrazinecarbothioamide (III)**

To a mixture of equimolar amount of 3-chloro-4-fluorobenzaldehyde (0.01 mol) and thiosemicarbazide (0.01 mol) in absolute ethanol (25 mL), 2-3 drops of acetic acid were added and content of the flask was refluxed for 2.0 h. After completion of the reaction monitored by TLC, the content of the flask was reduced to half by destillation and left overnight for crystallization. The crystalline mass obtained was filtered off, washed with water, dried, and recrystallized from ethanol.

**Synthesis of 5-(3-chloro-4-fluorophenyl)-1, 3, 4-thiadiazol-2-amine (IV)**

Thiosemicarbazone (**III**, 0.005 mol) obtained above was suspended in 300 mL distilled water in a 1000 mL beaker. Ferric chloride (0.15 mol) in 300 mL distilled water was added to it. The reaction mixture was stirred for one hour at 80-90°C and filtered while hot. A mixture of citric acid (0.11 mol) and sodium citrate (0.05 mol) was added to the filtrate and stirring was continued for 15 min. After cooling the whole solution, it was taken in a bigger vessel (to account for the increase in volume) and neutralized with 10% aqueous ammonia. The precipitate which separated out was filtered and recrystallized from 25% aqueous ethanol to give pure product.

**Synthesis of the title compounds (VIa-j)**

To a mixture of compound **V** (0.003 mol) and various substituted amine (0.003 mol) in 20 mL of absolute ethanol, 1 mL of triethylamine (TEA) was added and the whole was refluxed for 12–15 h. After completion of the reaction, content of the flask was reduced to half by distillation and left overnight. The crystalline mass obtained was filtered off, washed with water, dried, and recrystallized from ethanol to give the title compounds (**VIa-j**).

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(methylamino)acetamide (VIa)**

Yield 65%, m.p. 128°C, $R_f$ 0.63, *C* Log P 2.69; IR (KBr, $cm^{-1}$): 3323 (NH), 2897 (CH), 1556 (C=O), 1489 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.91 (s, 1H), 7.59-7.61 (d, 1H, *J* = 8.0 Hz), 6.53-6.41 (dd, 1H, *J* = 7.8, *J* = 2.3 Hz), 6.01 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.12 (s, 2H, $CH_2$), 2.26 (s, 3H, NH-$CH_3$), 0.97 (s, H, NH); ESI-MS m/z 300.74.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(cyclohexylamino)acetamide (VIb)**

Yield 61%, m.p. 140°C, $R_f$ 0.77, *C* Log P 4.66; IR (KBr, $cm^{-1}$): 3326 (NH), 2885 (CH), 1568 (C=O), 1475 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.79 (s, 1H), 7.45-7.47 (d, 1H, *J* = 7.6 Hz), 6.55-6.39 (dd, 1H, *J* = 8.1, *J* = 2.6 Hz), 6.14 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.15 (s, 2H, $CH_2$), 1.57 (m, 11H, cyclohexyl), 0.88 (s, 1H, NH); ESI-MS m/z 368.09.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(phenylamino)acetamide(VIc)**

Yield 59%, m.p. 119°C, $R_f$ 0.73, *C* Log P 4.18; IR (KBr, $cm^{-1}$): 3341 (NH), 2908 (CH), 1596 (C=O), 1481 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.95 (s, 1H), 7.61-7.63 (d, 1H, *J* = 8.0 Hz), 7.33-7.01 (dd, 1H, *J* = 8.3, *J* = 2.5 Hz), 6.95-6.54 (m, 5H, phenyl), 6.23 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.61 (bs, 1H, NH-phen., $D_2O$-exchangeable), 4.32(s, 2H, $CH_2$); ESI-MS m/z 362.04.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(p-tolylamino)acetamide (VId)**

Yield 70%, m.p. 133°C, $R_f$ 0.69, *C* Log P 4.64; IR (KBr, $cm^{-1}$): 3337 (NH), 2898 (CH), 1578 (C=O), 1475 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.81 (s, 1H), 7.58-7.60 (d, 1H, *J* = 8.2 Hz), 7.54-7.40 (dd, 1H, *J* = 7.0, *J* = 2.1 Hz), 6.91-6.50 (m, 4H, phenyl), 6.11 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.47 (bs, 1H, NH-phen., $D_2O$-exchangeable), 4.08 (s, 2H, $CH_2$), 2.26 (s, 3H, $CH_3$-phen.); ESI-MS m/z 376.06.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(4-chlorophenylamino)acetamide (VIe)**

Yield 63%, m.p. 129°C, $R_f$ 0.57, *C* Log P 4.97; IR (KBr, $cm^{-1}$): 3378 (NH), 2928 (CH), 1585 (C=O), 1488 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.88 (s, 1H), 7.78-7.80 (d, 1H, *J* = 7.8 Hz), 7.65-7.51 (dd, 1H, *J* = 7.6, *J* = 2.2 Hz), 6.98-6.25 (m, 4H, phenyl), 6.13 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.86 (bs, 1H, NH-phen., $D_2O$-exchangeable), 4.16 (s, 2H, $CH_2$); ESI-MS m/z 396.00.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(4-fluorophenylamino)acetamide (VIf)**

Yield 75%, m.p. 127°C, $R_f$ 0.88, *C* Log P 4.63; IR (KBr, $cm^{-1}$): 3381 (NH), 2934 (CH), 1589 (C=O), 1491 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.93 (s, 1H), 7.81-7.83 (d, 1H, *J* = 8.0 Hz), 7.69-7.50 (dd, 1H, *J* = 7.3, *J* = 1.9 Hz), 7.08-6.32 (m, 4H, phenyl), 6.18 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.93 (bs, 1H, NH-phen., $D_2O$-exchangeable), 4.21 (s, 2H, $CH_2$); ESI-MS m/z 380.03.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(4-methoxyphenylamino)acetamide (VIg)**

Yield 67%, m.p. 138°C, $R_f$ 0.83, *C* Log P 4.00, IR (KBr, $cm^{-1}$): 3345 (NH), 2906 (CH), 1552 (C=O), 1466 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.72 (s, 1H), 7.65-7.63 (d, 1H, *J* = 7.5 Hz), 7.54-7.33 (dd, 1H, *J* = 7.9, *J* = 2.4 Hz), 6.98-6.79 (m, 4H, phenyl), 6.12 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.61 (bs, 1H, NH-phen., $D_2O$-exchangeable), 4.17 (s, 2H, $CH_2$), 3.39 (s, 3H, -$OCH_3$); ESI-MS m/z 392.05.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(piperidin-1-yl)acetamide (VIh)**

Yield 72%, m.p. 115°C, $R_f$ 0.93, *C* Log P 3.24; IR (KBr, $cm^{-1}$): 3328 (NH), 2879 (CH), 1541 (C=O), 1452 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.61 (s, 1H), 7.24-7.22 (d, 1H, *J* = 7.7 Hz), 6.43-6.20 (dd, 1H, *J* = 7.4, *J* = 2.05 Hz), 6.08 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.15 (s, 2H, $CH_2$), 2.88-2.72 (m, 5H, piper.), 2.52-2.38 (m, 4H, piper.), 1.88 (s, 1H, NH); ESI-MS m/z 354.07.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-morpholinoacetamide (VIi)**

Yield 55%, m.p. 122°C, $R_f$ 0.86, *C* Log P 1.69; IR (KBr, $cm^{-1}$): 3336 (NH), 2883 (CH), 1556 (C=O), 1478 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.72 (s, 1H), 7.31-7.29 (d, 1H, *J* = 7.8 Hz), 6.49-6.25 (dd, 1H, *J* = 7.6, *J* = 2.2 Hz), 6.15 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.19 (s, 2H, $CH_2$), 3.97 (s, H, NH), 2.95-2.81 (m, 4H, morph.), 2.63-2.33 (m, 4H, morph.); ESI-MS m/z 356.05.

***N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]-2-(4-methylpiperazin-1-yl)acetamide (VIj)**

Yield 79%, m.p. 130°C, $R_f$ 0.65, *C* Log P 2.12; IR (KBr, $cm^{-1}$): 3331 (NH), 2878 (CH), 1549 (C=O), 1475 (C=C); $^1$H-NMR (300 MHz, $CDCl_3$, δ, ppm): 7.69 (s, 1H), 7.28-7.26 (d, 1H, *J* = 7.6 Hz), 6.41-6.17 (dd, 1H, *J* = 7.5, *J* = 2.3 Hz), 6.10 (bs, 1H, NHC=O, $D_2O$-exchangeable), 4.13 (s, 2H, $CH_2$), 3.86 (s, H, NH), 2.75-2.28 (m, 8H, piperazine), 1.86 (s, 3H, $CH_3$); ESI-MS m/z 369.08.

## Pharmacology

### Anticonvulsant activity

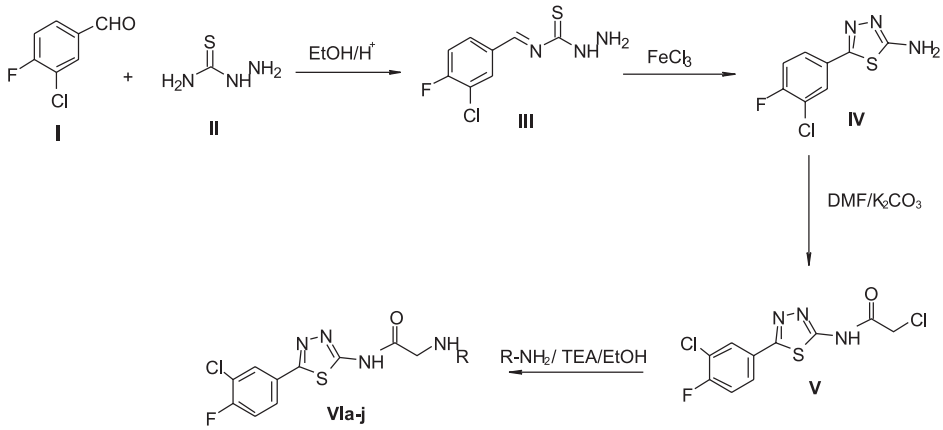
The investigations were conducted on Swiss albino mice of either sex (25-30 g). Food and water were withdrawn prior to the experiments. All the compounds (**VIa-j**) were dissolved in propylene glycol. Initially, all compounds were administered *i.p.* at doses of 30, 100, 300 mg/kg to mice. Activity was established using the MES and scPTZ tests according to the protocol (15, 16) by Antiepileptic Drug Development Program (ADD), Epilepsy Branch, National Institute of Health, Bethesda, MD, USA.

### Maximal electroshock seizure (MES) test

Mice were prescreened 24 h before the testing of the title compounds (**VIa-j**) by delivering maximal electroshock 50 mA; 60 Hz and 0.2 s duration by means of corneal electrodes.. A drop of 0.9% sodium chloride was instilled in each eye prior to the application of electrodes in order to prevent death of the animal. Abolition of hind limb tonic extensor component of the seizure in half or more of the animals is defined as protection.

### Subcutaneous pentylenetetrazol test (*sc*PTZ)

The *sc*PTZ test utilized a dose of pentylenetetrazol 70 mg/kg. This produced clonic seizures lasting for a period of at least 5 s. The test compounds were administered at the three graded doses i.e., 30, 100 and 300 mg/kg, *i.p.* At the anticipated




Scheme 1. Synthetic route of the title compounds (**VIa-j**)

time, the convulsant was administered subcutaneously. Animals were observed over a 30 min period. Absence of clonic spasm in half or more of the animals in the observed time period indicated a compound's ability to abolish the effect of pentylenetetrazol on seizure threshold.

**Neurotoxicity studies**

***Rotorod test***

The minimal motor impairment was measured in mice by the rotorod test (17). The mice were trained to stay on an accelerating rotorod of diameter 3.2 cm that rotates at 10 rpm. Trained animals were given *i.p.* injection of the test compounds in doses of 30, 100 and 300 mg/kg. Neurotoxicity was indicated by the inability of the animal to maintain equilibrium on the rod for at least one minute in each of the three trials.

***Ethanol potentiation test***

Mice were treated with the test compound and 1 h later with ethanol 2.5 g/kg *i.p*. This dose of ethanol did not induce lateral position in the control animals. The number of animals that were in the lateral position after receiving ethanol in each group was determined (18).

**Hepatotoxicity**

***Liver enzyme estimation***

Adult male albino rats (150-175 g) of Wistar strain were used to find out the toxic effects, if any, of the synthesized compounds on liver. The biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) were estimated by the Reitman and Frankel's method (19) while alkaline phosphatase (ALP) was measured by using King's method (20).

## RESULTS AND DISCUSSION

**Chemistry**

The compounds (**VIa-j**) were synthesized according to the route shown in Scheme 1. The synthesis of (*E*)-*N*-(3-chloro-4-fluorobenzylidene) hydrazine carbothioamide (**III**) was involved in a reaction between 3-chloro-4-fluorobenzaldehyde and thiosemicarbazide in the presence of ethanol and acetic acid. In the second step, cyclization of compound (**III**) takes place in the presence of ferric chloride resulting in 1,3,4-thiadiazole synthesis. Synthesis of 2-chloro-*N*-[5-(3-chloro-4-fluorophenyl)-1,3,4-thiadiazol-2-yl]acetamide (**V**) was performed by adding chloroacetyl chloride to a mixture of substituted 1,3,4-thiadiazole (compound **IV**). Similarly, the title compounds (**VIa-j**) were synthesized by reacting compound (**V**) with various substituted phenyl/cyclohexyl/amines in the presence of triethylamine (TEA). The outline of the reactions is shown in Scheme 1. All the synthesized compounds were well characterized by elemental analysis and spectroscopic data. The elemental analyses results for C, H and N were found within ± 0.4% of the theoretical value. In

Table 1. Anticonvulsant and neurotoxicity data of compounds (**VIa-j**).

| Compound | MES | | scPTZ | | Neurotoxicity | | Ethanol potentiation |
|---|---|---|---|---|---|---|---|
| | 0.5 h | 4 h | 0.5 h | 4 h | 0.5 h | 4 h | |
| **VIa** | 300 | 300 | X | X | X | X | X |
| **VIb** | 30 | 100 | 300 | (–) | 300 | (–) | 300 |
| **VIc** | 100 | 100 | 100 | 300 | 300 | (–) | (–) |
| **VId** | 100 | 300 | (–) | (–) | (–) | (–) | 300 |
| **VIe** | 30 | 100 | 100 | 300 | 300 | 300 | (–) |
| **VIf** | 100 | 300 | (–) | 300 | (–) | (–) | (–) |
| **VIg** | 30 | 300 | (–) | 300 | (–) | (–) | (–) |
| **VIh** | 300 | 300 | X | X | X | X | X |
| **VIi** | (–) | (–) | X | X | X | X | X |
| **VIj** | 300 | 300 | X | X | X | X | X |
| Phenytoin | 30 | 30 | (-) | (-) | 100 | 100 | X |

Dose of 30, 100 and 300 mg/kg were administered *i.p*. The figures indicate the minimum dose whereby bioactivity was demonstrated in half or more mice. The (-) indicates an absence of activity at maximum dose administered (300 mg/kg). The (X) indicates not tested.

Table 2. Enzyme estimation of the most active compounds.

| Compound | ALP (KA unit/dL) (Mean ± SEM) | SGOT (IU/L) (Mean ± SEM) | SGPT (IU/L) (Mean ± SEM) |
|---|---|---|---|
| **VIb** | 30.43 ± 1.62 | 43.37 ± 2.04 | 45.54 ± 1.23 |
| **VIc** | 38.79 ± 0.98** | 36.89 ± 1.05 | 40.94 ± 0.96 |
| **VId** | 31.97 ± 1.06 | 48.21 ± 1.71 | 51.27 ± 1.98 |
| **VIe** | 26.31 ± 1.17 | 37.21 ± 1.37 | 44.67 ± 1.40 |
| **VIf** | 24.97 ± 1.81 | 50.37 ± 2.01 | 51.99 ± 1.05 |
| **VIg** | 36.07 ± 1.59 | 54.28 ± 1.89 | 57.51 ± 1.91 |
| Control | 23.5 ± 1.70 | 29.57 ± 1.65 | 33.20 ± 0.85 |

The concentrations of SGPT and SGOT are expressed in international unit per liter whereas for alkaline phosphatase, the concentration is expressed as KA unit per deciliter. Number of animals tested (n = 6). Significantly different from control **$p < 0.01$. The mean ± SEM values were calculated using ANOVA followed by Dunnett's multiple comparison test.

the IR spectra, absorption bands for N-H, C-H, C=O and C=C were found in the region of 3381-3323, 3934-2878, 1596-1541 and 1491-1452 $cm^{-1}$, respectively. The $^{1}H$ NMR spectra showed three distinct aromatic zones of multiplet at δ values 6.65-7.93 ppm. A broad singlet was assigned for N-H attached with 1,3,4-thiadiazole.

**Anticonvulsant activity**

Anticonvulsant evaluation of compounds (**VIa-j**) in mice utilizing MES and scPTZ models are summarized in Table 1 together with the neurotoxicity data. Among the tested compounds of the series, **VIb, VIe** and **VIg** exhibited fifty percent or more protection at a dose of 30 mg/kg after 0.5 h and have shown activity comparable to phenytoin. Out of these three compounds, **VIb** and **VIe** were also active after 4 h at a dose of 100 mg/kg body mass. This shows the rapid onset and long duration of action of these compounds at a comparatively low dose. Compounds **VIc, VId** and **VIf** showed protection at a dose of 100 mg/kg body mass after 0.5 h. These compounds were also active after 4 h but at a higher dose, 300 mg/kg body mass, except compound **VIc** which was active after 4 h at the same dose i.e., 100 mg/kg body mass. This shows the ability of these compounds to prevent spreading of seizures. Compound **VIi** is devoid of activity in MES test whereas the rest of the compounds have very low activity.

Only those compounds were chosen for chemoshock activity utilizing scPTZ animal model that showed the highest to moderate activity against MES test. Therefore, compounds **VIb**, **VIc**, **VId**, **VIe**, **VIf** and **VIg** were subjected to scPTZ test. Compounds **VIc** and **VIe** showed significant activity while compounds **VIb**, **VId**, **VIf** and **VIg** exhibited low or no activity.

The undesired side effect i.e., neurotoxicity of highly and moderately active compounds were evaluated by conducting the rotorod and ethanol potentiation tests and the results are expressed in Table 1. Three compounds **VIb**, **VIe** and **VIg** that exhibited encouraging anti-MES activity were found safe or to possess very low neurotoxicity. Two anticonvusant active compounds, **VIb** and **VId** showed very low motor impairment in ethanol potentiation tests. The other active compounds successfully passed the rotorod and ethanol potentiation test.

Compounds **VIb**, **VIc**, **VId**, **VIe**, **VIf** and **VIg** were selected for the liver enzyme analysis as these compounds were found potent in anticonvulsion tests. Exposure to high concentrations of these compounds (300 mg/kg) may result in its accumulation in the liver and, in turn, to alterations in the liver function. Transaminases (SGOT and SGPT) are intracellular enzymes, released into the circulation after damage and necrosis of hepatocytes. Alkaline phosphatase (ALP) is a membrane-bound enzyme related to the transport of various metabolites and is considered a sensitive biomarker for liver disease. Liver enzyme estimation of the above mentioned compounds was carried out and the results are shown in Table 2. Results are expressed in international unit per liter (as the mean ± SEM) for SGOT and SGPT and KA unit per deciliter for alkaline phosphatase, with six animals in each group whereas **$p < 0.01$ is significantly different from control. It was observed that all the values were comparable to the control and the changes seen, were not significant except in case of compound **VIc** that showed an elevated alkaline phosphatase value ($p < 0.01$). Hence it can be concluded that except compound **VIc**, the other tested compounds do not possess any adverse affect on liver or bone tissues.

The *C* log P data for all the title compounds (**VIa-j**) were calculated using ACD lab version 8.0. A majority of the compounds i.e., **VIa**, **VIb**, **VIc**, **VId**, **VIe**, **VIf**, **VIg**, **VIh**, and **VIj** have *C* Log *P* value above 2 except compound **VIi** which has *C* Log P value 1.69. It was suggested in the literature that a *C* Log P value of at least 2.0 is required for a drug to cross the blood brain barrier (21, 22). The *C* Log P data were in agreement with the aforementioned hypothesis (except compound **VIg**) and it was observed that those compounds which had C Log P values above 2 exhibited significant anticonvulsant activity.

In structure activity relationship, it was noticed that one of the major contributing factors which regulate the anticonvulsant activity was the different substitutions in the aromatic ring, and particularly halogen substitutions (**VIe** and **VIf**) showed an increase in the potency in MES screen**.** Furthermore, the presence of a bulkier electron donating methoxy group at para position of the phenyl ring i.e., compound **VIg**, exhibited a paradoxical result with the lipophilicity hypothesis. An increase in anticonvulsant activity was observed in methoxy derivative (compound **VIg)**, even though methoxy derivative was less lipophilic than the methyl (compound **VId)** and the unsubstituted (compound **VIc)** derivatives of phenyl ring. The same lipophilic hypothesis may be applied for highly active compound **VIb** (30 mg/kg) which contains cyclohexyl group. Replacing carbon atom from cyclohexyl ring with any heteroatom caused a decrease in activity as observed in compounds **VIh**, **VIi** and **VIj**.

In conclusion, it can be said that the present series of acetamide derivatives display an encouraging anticonvulsant activity with lower neuro- and hepatotoxicity. These compounds can be considered as lead molecules for future investigations.

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