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REVIEW

NEW TRENDS IN THE TREATMENT OF NICOTINE ADDICTION

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Abstract: The aim of this study was to discuss the therapeutic substances used to treat nicotine addiction, not registered in Poland. This paper presents the results of the latest clinical trials and the possibility of their use in the treatment of nicotine addiction. The first two discussed drugs – clonidine and nortriptyline are recommended by clinical practice guidelines AHRQ (Agency for Healthcare Research and Quality) as the substance of the second line in the fight against addiction. Nortriptyline belongs to tricyclic antidepressants. Its mechanism of action is the inhibition of the reuptake of norepinephrine. It is suggested as the antagonist of activity of nicotinic receptors. The results confirm its efficacy in the treatment of nicotine addiction, but many side effects limit its use. Clonidine acts presumably by inhibition of sympathetic hyperactivity characteristic of symptoms associated with nicotine rehab. The remaining compounds under discussion, such as: venlafaxine, fluoxetine, moclobemide and rimonabant, are not registered in any country with an indication to use in the treatment of nicotine addiction, however, due to the mechanism in which they act, the possibility of their use in the treatment of this disease is considered. The possibility of using anxiolytics such as: buspirone, diazepam, meprobamate and β -blockers: metoprolol and oxprenolol is also considered in order to treat the anxiety appearing as one of the symptoms of abstinence. An interesting proposal to combat nicotine addiction are vaccines – NicVAX, CYT002-NicQb and TA-NIC. Currently, they are in clinical phase I and II of their development. Their operation would be based on the induction of specific antibodies that bind nicotine in the plasma, thus prevent it reaching the nicotinic receptors. Preliminary results confirm the possible positive effects in the prevention and treatment of nicotine addiction.

Keywords: nicotine addiction, nortriptyline, clonidine, bupropion, cannabinoid receptor antagonists, anxiolytic drugs, inhibitors of cytochrome CYP2A6, nicotine vaccines

According to the World Health Organization studies, in Poland, percentage of daily smoking women over 20 years old is 26%, and percentage of men in the same age is 43%. Epidemiological calculations showed, that in the year 2000 smoking was the reason of ca. 69 thousands of deaths in Poland (including: 57 thousands of men and 12 thousands of women) (1).

It has been proven, that smoking tobacco is the best known factor of many diseases. To the health consequences connected with tobacco smoking belong: diseases of circulation system, chronic obstructive pulmonary disease, hypertension, atherosclerosis and tumors. The big problem is also passive smoking, result of which is predominantly increased risk of lung cancer and ischemic heart dis-

ease (2–4). Epidemiological studies, referred to the effects of smoking on human health, which were conducted, confirm rightness of tobacco dependence fighting.

In Poland, one of the most frequently used questionnaire, which helps to discern tobacco addiction is Fagerstöm test (2, 5). It's used to measurement of pharmacogenic component of nicotine addiction. Maximal number of points, which can be obtained from this test is 10. Result equal or higher than 7 indicates probable pharmacological addiction. In this case, physician should consider introduction of pharmacological treatment (4, 6).

Currently, it is believed that doctor – patient conversation and motivating the patient by doctor to stop smoking, and stay in abstinence as long as pos-

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sible has a great importance. But besides „conversation”, pharmacological treatment is also used, as a help for patient in smoking quitting process, especially to relieve withdrawal symptoms.

In Poland, first choice drugs, which are used in nicotine addiction are nicotinic replacement therapies and bupropion SR (3, 4). Quite popular drugs also used in fighting the addiction are varenicline and cytisine. The choice of drug is usually a result of doctors experience in use of certain product, occurrence of indications and preferences and individual patient characteristics (3).

Second line drugs in America, but not registered for nicotine treatment in Poland, are clonidine and nortriptyline. Despite the demonstrated effectiveness of treatment nicotine addiction, the use of them is limited, mainly due to side effects, which are occurring more often than for the first-line drugs). This drugs are not approved by FDA (Food and Drug Administration) as drugs used for nicotine treatment, but are recommended by AHRQ (Agency for Healthcare Research and Quality) in some cases: when using of first-line drugs (individually or in the therapy complexes) is not bringing effects or they are contraindicated (4, 7–10).

Nortriptyline

It is a drug belonging to the tricyclic antidepressants. Its effect in the treatment of nicotine addiction results from inhibition of the reuptake of norepinephrine. It has a relatively high affinity for both the serotonin receptors and serotonin conveyors, as well as dopamine transporters. There is evidence that nortriptyline acts as a weak antagonist of nicotinic receptors, suggesting a potential mechanism of action in the fight against nicotine addiction (10–12).

The effectiveness of nortriptyline as a medication that helps to stop smoking were evaluated in two double-blind placebo-controlled studies. In each of these studies were involved approximately two hundred people. From these studies were excluded those, who suffered from depression after their inclusion in the study, in order to eliminate the effects of non-action of the antidepressant nortriptyline. It was found that the use of nortriptyline statistically increases the number of people who stopped smoking in comparison to the number of people who stopped smoking using placebo. There was two-fold increase in the smoking cessation one year after the start of therapy, resulting from the use of nortriptyline *versus* placebo. In subsequent studies, a fivefold increase was found. These studies involving 413 test persons, suggest that nortriptyline may be helpful in

quitting smoking (13). Other studies have shown that nortriptyline combined with transdermal nicotine system increases the frequency of stop smoking over the average observed when using only nicotine transdermal patches (14). In the treatment of nicotine dependence by nortriptyline, dosages applied were between 25 mg and 100 mg per day (15).

Nortriptyline, an antidepressant medication from the group of tricyclics, may increase the risk of suicide (4). Other side effects in the use of tricyclic antidepressants may be a block of: muscarinic receptors, (which results in: dry mouth, dim vision, constipation, urinary retention), histamine H1 receptors (which results in: sedation, sleepiness, weight gain), and α 1-adrenergic receptors (which results in orthostatic hypotension) (4, 16, 17). Although nortriptyline may have any of the these side effects mentioned above, it is considered as one of the least sedative tricyclic antidepressants, as well as is rarely associated with orthostatic hypotension (17).

Clonidine

Clonidine is the second of the drugs recommended in the treatment of second-line treatment of nicotine addiction by AHRQ clinical practice guidelines. Furthermore, a drug is used to treat withdrawal symptoms occurring during treatment of opioid and alcohol addiction. Its effects are probably related to the reduction of sympathetic overactivity, characteristic for withdrawal symptoms (11). Clonidine is an agonist of α 2-adrenergic receptors and is usually used as a medicine against hypertension. In connection with the possibility of the emergence of withdrawal reactions, characterized by a sudden increase in blood pressure, which can lead to hypertensive crisis, the elimination of clonidine treatment has to be done slowly (11).

Clonidine activity was evaluated in three meta-analyses, which rated the results of research on the impact of clonidine on smoking cessation. One of the meta-analyses based on the conclusions of nine randomized controlled trials, double-blind, showed that clonidine is helpful in increasing the percentage of people's stop smoking (OR 2.36, 95% CI: 1.69–32.8) (18). The second meta-analysis has led to a similar conclusion: OR 2.0 (95% CI: 1.3–3.0) (19). Third among the meta-analyses serving as a basis for AHRQ recommendations also showed that clonidine increases the percentage of people who stop smoking to a much greater degree than placebo (OR 2.1, 95% CI: 1.4–3.2) (16). On the basis of these meta-analyses it was concluded that clonidine is an effective drug to help quit the habit in some

populations. So far it is not clear which patients most effectively react to treatment with clonidine. Studies suggest that clonidine is effective in women and ineffective in men, while other studies have shown similar effects of clonidine in both genders (20).

The use of clonidine is contraindicated in pregnant women, and among people inclined to risky behavior. The most common side effects of clonidine include: dry mouth, drowsiness, dizziness, sedation, above average fatigue or tendency to constipation (4, 11). During treatment, however, may be disclosed much heavier symptoms that clinicians and patients should be aware of, such as: allergic reactions, slow heartbeat and sometimes an increase or decrease in blood pressure (11).

In spite of this extensive research, the role of clonidine as smoking cessation aid is still unclear. In view of these uncertainties, the occurrence of side effects and the possibility of withdrawal reactions, clonidine is considered as a second-line help in quit smoking.

Antidepressants different than bupropion and nortriptyline

Recent studies suggest that smoking results from desire of self-compensation of mood disorders through administration of substance, which stimulates dopamine release and neurons connected with reward system. There are ongoing researches on possibility of using substances such as: venlafaxine, fluoxetine and moclobemide in treatment of addiction (11, 15). Results of current studies confirm that effectiveness of this drugs is comparable with placebo and nicotine replacement therapy (15).

The mechanism of pharmacological action, which is a condition for effectiveness of individual antidepressants is unclear. For example, nortriptyline has high affinity to norepinephrine and serotonin transporters, but bupropion has relatively low affinity. On the other hand, paroxetine, for which effectiveness as a drug which may be used in therapy of addiction wasn't demonstrated, has similar to bupropion and higher than nortriptyline affinity to dopamine transporters. According to what stays above, antidepressants action can not be explained only by analyzing of interaction with monoaminergic receptors (11).

Cannabinoid receptor antagonists

Endocannabinoids and their receptors CB1 and CB2, which are located on surface of neurons, are forming endocannabinoid system. This structure is responsible for regulation of synthesis and release of

γ -aminobutyric acid, which controls synthesis of dopamine (reward system). It has been shown that for addicted persons that system is deregulated and that receptors CB1 are hyperactive. These receptors play a role in cerebral system of reward, control of food intake, substance abuse and habitual behavior (11). Rimonabant is a selective antagonist of cannabinoid receptor CB1 (which is located in brain, adipose tissue, skeletal muscles and liver (11)). In preclinical studies, drug intake resulted in reducing the amount of ingested nicotine. The efficacy of drug was assessed in Cochranes systematic review, based on reliable, randomized two clinical trials of third phase. Higher possibility of stop smoking and maintenance of abstinence after one year was observed in group of patients to which rimonabant was given in the dose of 20 mg/day, compared with placebo (OR: 1.46, 95% CI: 1.16–1.85) (21).

In clinical trials, clear evidence about rimonabant effectiveness was not observed. Usage of rimonabant probably contribute to significant reduction of weight gaining, after quit from smoking. The most common side effects of rimonabant are diarrhea and upper respiratory tract infection. The impact of rimonabant on the cardiovascular system was not noticed so far. According to that, this drug seems to be safe and may be used in nicotine addictional treatment, with using it's preventing weight gain property, which is disruptive side effect in the process of quitting smoking for many addicted. However, the introduction of rimonabant as a drug used in nicotine dependence should require more studies (11).

Anxiolytic drugs

Suggestions about usage of these drugs in the treatment of nicotine dependence are due to the fact that nicotine has properties to reduce anxiety and tension. Anxiety may also be one of the symptoms that arise from abstinence. The use of anti-anxiety medication would be designed to reduce withdrawal symptoms. Suggested anxiolytics include: buspirone, diazepam, meprobamate, ondansetron and β -blockers (metoprolol and oxprenolol) (22, 23).

Inhibitors of cytochrome CYP2A6

In human body, ca. 70–80% of nicotine is metabolized to cotinine and this transformation is catalyzed by CYP2A6 enzymes. It was shown that polymorphic differences in formation of these enzymes are important in pharmacokinetics of nicotine and formation of dependence. Considering these data, we can conclude that inhibitors (specific

block) of CYP2A6 may be used in nicotine addiction treatment. There are suggestions about possibility of using them together with nicotine replacement therapy (NRT), which may increase the level of nicotine without changes in its dose (11, 24). It was observed that using strong inhibitors of CYP2A6 – methoxsalen and tranlycypromine together with nicotine chewing gum, a significant increase of levels of nicotine in plasma and reduction of the urge to smoke (25, 26) occurred.

Opioid receptor antagonists

Nicotine exposition is connected with activation of cholinergic nicotine receptors, resulting in a release of neurotransmitters (including endorphins). Their presence is associated with a reduction in anxiety and tension and the feeling of pleasure and relaxation. There are opinions that using of antagonists of opioid receptors, can reduce rewarding of nicotine action. In one study conducted on rats, it was demonstrated that opioid receptor antagonists, such as naloxone or naltrexone, reduce the number of cigarettes smoked, lower satisfaction with smoking and increase the likelihood of quitting smoking (27). This study suggests that opioid receptors can modulate the reinforcing effects of nicotine (28).

GABAergic drugs

Theoretically, GABA neurotransmission affecting drugs can reduce reinforcing effects of nicotine that can be helpful in fight against tobacco addiction (29). Proposed for this kind of action drugs are: vigabatrin, baclofen, gabapentin and tiagabine. Results of studies of these drugs show that there are neurobiological mechanisms through which GABA neurotransmission affecting drugs can be helpful in treatment of tobacco dependence. Unfortunately, until now, relatively few studies considering these drugs have been conducted. However, considering results of laboratory and preclinical studies, it may be possible that in the future, these drugs may be used in such treatment (11).

Mecamylamine and lobeline

Drugs contained in this group previously have already been assessed earlier in terms of their usefulness in the treatment of tobacco addiction. Both drugs are characterized by a low efficiency, and low side effect profile (11). Mecamylamine is a non-competitive antagonist of nicotinic cholinergic receptors. In theory, an antagonist should block the physiological effects of nicotine, including its reinforcing effect. Consequently, the use of mecamylamine should lead to a reduction in the desire to

smoke a cigarette (30). It was found in some cases that mecamylamine given smokers instead of decreasing, increases nicotine craving and may even tempt to reach for another cigarette (30, 31). There is evidence that mecamylamine is useful in treating nicotine dependence in certain “resistant” smokers. A limitation to its use are side effects such as: hypotension, dizziness and constipation (31).

Lobeline, along with nicotine, was one of the first drugs used in the treatment of nicotine dependence (32). Lobeline is the alkaloid and nicotine receptor agonist, obtained from the leaves of the bloated lobelli (*Lobelia inflata*). Starting from the thirties of the twentieth century, it was often used in the form of different preparations. A recent study on the effectiveness of lobeline in long-term treatment of addiction provides evidence proving that lobeline may be helpful in stopping smoking. Side effects of lobeline include: nausea, dizziness and vomiting. Tablets and pills containing lobeline can cause irritation of the throat (32).

Nicotine vaccine

Studies on the development of nicotine vaccines are now in progress (phase I and II clinical trials). The principle of operation is based on the fact that nicotine vaccines induce the production of antibodies, which can bind the particles in the plasma nicotine, preventing it to reach the call characteristic of receptors and the effect of smoking. In one of the study, rats were given the active vaccine or placebo, and 30 min later they were given nicotine at a dose of 0.03 mg/kg intravenously, corresponding to acceptance by smokers nicotine contained in two cigarettes (33). Compared with the control, the vaccine reduced the concentration of nicotine in the brain in dose dependent manner (65% decrease in the concentration at the highest doses). The use of vaccine prior to the administration of five doses of nicotine (corresponding to 10 cigarettes burn) over the period of 80 min also changed the distribution of nicotine to the brain (11). Potential mechanisms and clinical usefulness of vaccines is intriguing. On the one hand, thanks to anti-smoking vaccine smoking ceases to give pleasure, and it helps to break the addiction, but on the other hand, as a result of significant reduction or elimination of nicotine reaching the brain, some smokers will increase the dose of nicotine taken in order to provide commonly used (before treatment) doses.

The results of the studies so far have indicated the use of such vaccines in preventing relapse of addiction. They may also be used among adolescents as a preventative treatment for preventing

smoking. Undoubtedly, further studies are evaluating the potential benefits and ethical implications of such an intervention (34).

There are several companies conducting clinical trials of anti-nicotine vaccines. Among them are: Nabi (NicVAX [Nicotine Conjugate Vaccine]), Cytos (CYT002-NicQb), and Celtic Pharma (TA-NIC) (35). NicVAX vaccine consists of a hapten 3'-aminomethylnicotine, which was connected to protein A obtained from the *Pseudomonas aeruginosa* (11). Preclinical studies have shown that vaccination with NicVAX prevents nicotine to reach the brain and blocks the effects of nicotine, including effects that can lead to addiction. Clinical studies have shown that vaccination with NicVAX of smoking people who sincerely want to quit smoking in conjunction with the patient's motivation to quit smoking and abstinence as long as possible by a physician, has significant beneficial effects for quitting smoking. In the second phase of clinical trials, 68 smokers not interested in quitting are given three different doses of the vaccine or placebo (36, 37). The vaccination took place on the following days of the therapy: 0, 28, 56 and 182. The subjects were monitored for a period of 38 weeks. The results showed that the vaccine is safe to use and well tolerated. In addition, although there was no attempt on its effectiveness, it has been observed that the ratio of 30-day abstinence was significantly different among the doses, and the highest rate was characterized by the highest dose of vaccine administration. There was no increase in the number of test persons of cigarettes smoked in order to compensate for the nicotine neutralization effect was observed among the patients. In November 2011, the results of phase III of clinical trials with NicVAX® in which the treatment not meet the primary endpoint were published. Further studies of phase II of the trials with NicVAX in combination with varenicline also fail to meet the primary endpoint. Currently, the clinical trials concerning the NicVAX vaccine have been discontinued (38).

The vaccine CYT002-NicQb is based on a virus-like particle obtained by a recombination of the bacteriophage Qb mantle protein. In the first phase of clinical trials, two intramuscular injections or a placebo were given to a group of 40 healthy and non-smoking volunteers in four-week intervals (39). Specific IgM antibodies began to appear after 7 days and IgG after 14 days. The level of antibodies has been increased after the second injection. It has been shown that the vaccine is safe and well tolerated. In phase II of the clinical trials (double-blind sample), 340 people addicted to cigarette smoking were vac-

inated using the said vaccine 5 times in one-month intervals (40). Among the subjects showed a negligible abstinence, lasting 2–6 months, slightly higher compared to the abstinence people used a placebo. A significant effect was found among a group of people who have demonstrated high levels of antibodies. Moreover, it was not observed that people who re-started smoking compensated for the nicotine's neutralization effect by increasing the number of cigarettes smoked (40).

Immunotherapeutic vaccine TA-NIC has been evaluated in two phase study conducted in the UK, studying 120 smokers. During the study there were no adverse side effects. The vaccine's effectiveness is comparable to the placebo's effect (41, 42).

In summary, it can be stated that the nicotine vaccines may be effective in the treatment of tobacco addiction, however, the approval of these products probably will take several years.

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RECENT PHARMACOLOGICAL ADVANCEMENTS IN SCHIFF BASES: A REVIEW

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Abstract: Schiff bases are the biologically privileged scaffolds in organic chemistry, commonly synthesized from the condensation reaction of carbonyl functional group with amines. Naturally occurring and synthetically prepared Schiff bases are active molecules with many pharmacological activities like antibacterial, anti-cancer, anti-fungal, anti-malarial, antioxidant and many more. This review article summarizes pharmacological developments in the recent few years and gives a brief overview of their therapeutic potential.

Keywords: aldehydes, antibacterial, antifungal, ketones, antiinflammatory, anti-cancer, antioxidant, Schiff bases

“Schiff bases” were named after the German chemist Hugo Schiff and are produced by reacting the aldehyde or ketone with primary amines (1), they can be used as reactive intermediates for the synthesis of many natural products (2). Schiff bases are reported to show a wide range of pharmacological activities and are used as antimicrobial agents with the activities including antibacterial, anti-fungal, anti-malarial and anti-viral agents as well as the anti-inflammatory, antioxidant and anti-cancerous agents (3, 4). Pharmacological activities attributed by Schiff bases are mainly due to characteristic C=N functionality (Figure 1). Synthetically, condensation of amine with carbonyl compound occurs under reflux conditions with complete removal of water molecule formed in the system by using molecular sieves; removal of water can also be done by using well known dehydrating solvents *in situ* i.e., trimethylorthoformate or tetramethylorthosilicate (1).

Other methods has also been reported for synthesis of Schiff bases, that involve the use of Lewis or Bronsted-Lowry acids, some common are ZnCl₂, TiCl₄, MgSO₄-PPTS, Ti(OR)₄, alumina, H₂SO₄, NaHCO₃, MgSO₄, Mg(ClO₄)₂, H₃CCOOH,

Er(OTf)₃, P₂O₅/Al₂O₃ and HCl, as catalyst (1, 3–6). New cost effective and efficient methods including, microwave accelerated, solvent free synthesis, and solid state synthesis are also being used and reported for the synthesis of Schiff bases and their metal complexes (1, 7, 8)

This brief review summarizes the pharmacological importance of different synthetic Schiff bases derived from some natural products or from commercially available precursors and also suggest the future perspectives of potential research areas.

PHARMACOLOGICAL SIGNIFICANCE OF SCHIFF BASES

Biologically active molecules, Schiff bases, are known to show a variety of pharmacological activities. The literature available and used in this review has been summarized in Table 1.

Antimicrobial activities

Anti-plasmodium activity

Malaria, a disease caused by genus *Plasmodium*, claims approximately one million death tolls annually and is a serious threat to developing countries. World Health Organization report-

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ed over 500 million affected people, 90% are children in sub-Sahara, Africa. A female mosquito *Anopheles* is responsible for the cause of malaria, widely spread in more than 100 countries (9).

Plasmodium falciparum (*P. falciparum*) is getting resistant against the available drugs in the market; therefore, there is a constant need for the introduction of new therapeutic agents to act against the disease. Schiff bases are the potential molecules,

which can be effective against the problem of drug resistance. In addition to the synthetic derivatives, ancistrocladidine having iminium group moiety, is a natural product produced by plants belonging to family Dioncophyllaceae and Ancistrocladiceae and is known as anti-malarial agent with activity against *P. falciparum* strains 3D7 and K1 (1). Moreover, metal complexes like ruthenium complexes of Schiff bases derived from aryl and ferrocyl group show activity against the *P. falciparum* strains (3). Schiff bases obtained by the condensation of 2,6-diarylsubstituted piperidin-4-ones with 7-chloro-4-hydrazinoquinoline have also been tested for anti-malarial activities and reported to show strong anti-malarial activity against the *P. falciparum* strains (10). Therefore, in the quest of new effective drug molecules against malaria, Schiff bases can be a potential avenue of research.

Table1. Literature of activities of Schiff bases.

No.	Activity	References
1	Anti-plasmodium	1, 3, 10
2	Antibacterial	1, 11–23
3	Anti-fungal	1, 17, 24–32
4	Anti-viral	1, 33–37
5	Anticancer	38–41
6	Antioxidant	42–46
7	Anti-inflammatory	47–51

Antibacterial activity

Drug resistance against available antibiotic drugs is also a fast growing issue that the modern world has to face in the coming years. This can

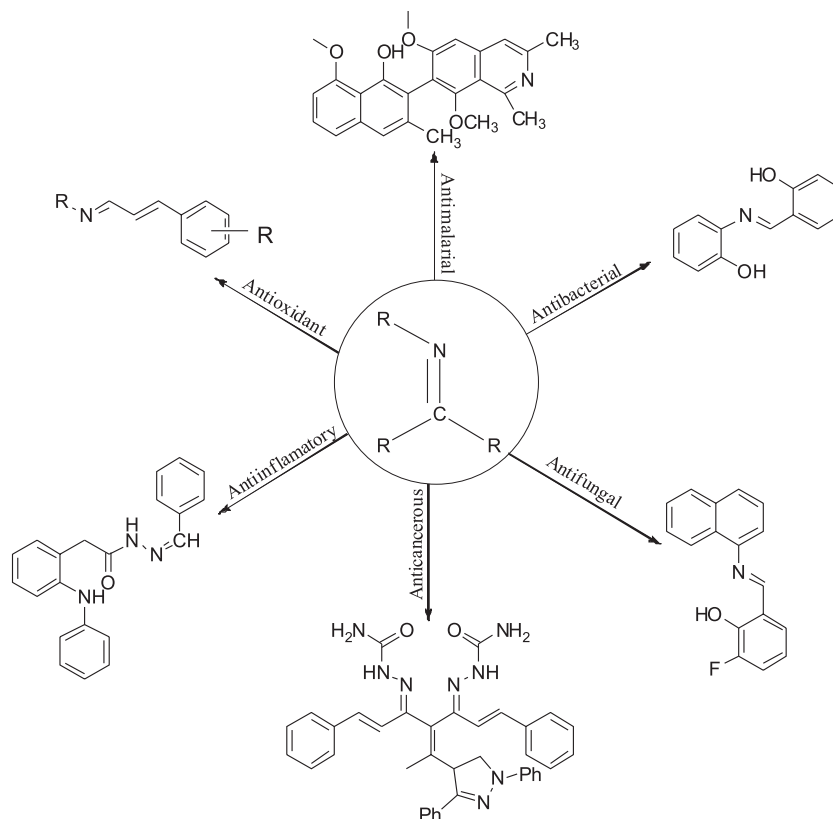


Figure 1. Pharmacologically active Schiff bases (1, 14, 29, 38, 43, 49)

potentially result in a dramatic increase in death rate and infectious diseases especially after accidental and surgical cases (11). Therefore, in order to cope with these problems, there is an immediate and constant need of new synthetic moieties with better and acceptable therapeutic index (12). Schiff bases have been considered the agents, which have more effective activity against the infectious bacteria, Schiff bases synthesized from 2-hydroxy-1-naphthaldehyde and α -amino acids (L-tyrosine, L-arginine, and L-lysine) and their manganese complexes have been reported to show excellent activity against the Gram positive and Gram negative strains of bacteria (13). Additionally, Schiff bases derived from salicylaldehyde show potent antibacterial activities, for example, *N*-(salicylidene)-2-hydroxyaniline has been reported to show a prominent activity against *Mycobacterium tuberculosis* (1), while Schiff bases of 5-chlorosalicylaldehyde show enhanced antibacterial activity against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Micrococcus luteus* (*M. luteus*) strains of bacteria (14). However, Schiff bases derivatives have also been reported to act as bacteriostatic agents e.g., Schiff bases of 2,4-dichloro-5-fluorophenyl are useful to stop the bacterial growth (1, 15). Moreover, Schiff bases bearing nitroimidazole moiety show good antibacterial activities against various bacterial strains (16). In addition, Schiff bases, derivatives of isatin, has been reported to show antimicrobial activity comparable with that of the standard drug sulfamethoxazole (17). Schiff bases synthesized from other substrates, including, morpholines, coumarins, *o*-phthaldehyde, aminothiazolylbromocoumarins, sulfonamides, acetophenones, crown ethers, amino acids and 2-aminophenol and 1,2,4-triazoles, were reported to show very low antibacterial activities (1, 4, 18–23).

Anti-fungal activity

Fungal infections are not limited to tropical areas but can also lead to increased risk of systemic infections, which may be life threatening (24). Factors for an increase in systemic fungal infections are geriatric patients, surgeries, AIDS, treatment of various tumors, transplantation of hard organs, hematopoietic stem cells and immunosuppressive treatment (25–27). Therefore, it is important to develop and formulate more effective and safe anti-fungal drugs, which can be effectively used in various medical conditions (28). Schiff bases have been reported to show good anti-fungal activity, e.g., Schiff bases of *N*-(salicylidene)-2-hydroxyaniline and from 3-fluorosalicylaldehyde are reported to show antifungal activities (1). However, transition

metal complexes of Schiff bases derived from *N,N*-ethylene (bis 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazine-1-yl)-quinoline-3-carboxylic acid reported to show higher antifungal activity than their precursor Schiff bases (29). Oxovanadium (IV) complexes of Schiff bases show more activity as compared to their ligand (30). In addition, chitosan Schiff bases have been reported to stop the growth of many fungal strains including *Colletotrichum humblenarium* and *Botrytis cinerea* (1, 31). Moreover, isatin based Schiff bases has been reported to show remarkable antifungal activity against various fungal strains like *Microsporium gypseum* and *Microsporium audouinii*, furthermore, isatin Schiff base derivatives also show anti-fungal activity against *Cryptococcus neoformans* (*C. neoformans*), *Epidermophyton floccosum* (*E. floccosum*) and *Candida albicans* (*C. albicans*) (1, 17, 32).

Antiviral activity

Presently, a large number of viral diseases are treated either adopting vaccination or by using antiviral drugs. Drug resistance reported in viral diseases is a serious issue for humanity; therefore, new therapeutic molecules are constantly required (33). Some common viral diseases like, influenza, rubella, small pox, chicken pox and polio can be controlled by vaccines administration, while viral diseases like *hepatitis 'C'* is still under the process of vaccine discovery (1, 34). Therefore, Schiff bases can play a vital role due to their reported antiviral nature. Schiff bases derived from isatin and bis-isatin are reported to show activities against different strains of viruses (1, 34, 35). Moreover, Schiff bases derived from prodrug abacavir (Ziagen) are reported to show good antiviral activity and trials revealed that they are potent lead molecules for further clinical use as anti-HIV therapy (1, 36). Furthermore, Schiff bases of 2-phenylquinazoline-4(3H)-one are reported to show antiviral activity against some strains of viruses like feline corona virus, influenza viruses, and herpes simplex virus type 1 and 2 (37). The antiviral potential of these Schiff bases is evident from reported literature and therefore more targeted research can help to discover and develop new potential lead compounds to use them as drug candidates.

Anticancer activity

Cancer is a disease which leads to death. More than 200 cancer types have been reported in the human body. Schiff bases obtained from coumarin and pyrazole aldehyde has been tested against cancerous cell lines and showed mild anti-cancerous

activities (38). Moreover, in another study, mono and bis-Schiff bases have been reported effective against five cancer cell lines (39). Furthermore, Schiff bases can effectively form complexes with transition metals and these metal complexes are reported to show good anticancer activities; Cu-complexes with vaniline Schiff bases (40) and 5-dimethyl-2-phenyl-4-[(pyridin-2-ylmethylene)-amino]-1,2-dihydro-pyrazol-3-one Schiff bases (41) has been reported for their anti-cancerous activities. Extensive literature is available on the effectiveness of Schiff bases against cancer cell lines, therefore, a more systematic and extensive research, both *in vitro* and *in vivo*, is suggested to extend their therapeutic use to alleviate the disease.

Antioxidant activity

Aging is an evident phenomenon that a human has to face. Production of reactive oxygen species (ROS) increases with the passage of time, in the human body and leads to many physiological disorders including cardiovascular diseases. Schiff bases and their metal complexes play an important role in the production of ROS (42) and therefore, can show antioxidant properties. Recently, Schiff bases of natural phenylpropene derived methoxylated cinnamaldehydes (43), and tin metal complexes have been reported for antioxidant activities (44). In a recent study on thymol and carvacrol Schiff base derivatives in 5 µg/mL concentration showed 60–90% inhibition for antioxidant activity (45). Moreover, Schiff bases of 2-oxoquinoline-3-carbaldehyde have been reported as excellent anti-oxidizing agents and their activity was comparable with the ascorbic acid used as standard (46). Literature reveals their effectiveness in the antioxidant behavior; therefore, more targeted research can possibly lead to their use in the therapy of various ailments.

Anti-inflammatory activity

Non-steroidal anti-inflammatory drugs (NSAIDs) are being used for the treatment of pain and perform their function by inhibiting the production of prostaglandins (PG), which are involved in many physiological activities (47, 48). Occasionally, these NSAIDs are not targeted for the particular enzyme involved in the biosynthesis of prostaglandins; therefore, for more targeted attack on the particular isozyme new effective molecules are required. Therefore, Schiff bases derived from 2-(2,6-dichloroanilino) (49) and 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one have been reported for excellent anti-inflammatory activities (50). Moreover, transition metal complexes of Schiff

bases containing aldose group have also been reported for anti-inflammatory activities (51). Further investigations are suggested for their preferential therapeutic use in sickness and accidental case of inflammation.

CONCLUSION

Schiff bases and their derivatives are a class of compounds with literature evident pharmacological importance and applications. Therapeutic spectrum is also wide and less explored for Schiff bases, therefore, a scientific approach is required to establish the structure activity relationships of these biologically and medicinally viable molecules. Concisely, Schiff bases are among the molecules which have therapeutic potential for the treatment of various human diseases.

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FUNCTIONS AND APPLICATIONS OF EXOSOMES

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Abstract: The cells continuously secrete a large number of different types of microvesicles including macro- and micro-molecules into the extracellular fluids. One of them are exosomes, which are nano-sized vesicles capable of transferring the DNAs, microRNAs, non-coding RNAs and lipids with or without direct cell to cell contact, so representing the novel way of intracellular communication. In this review, we tried to summarize the exosomal structure, composition, formation, and isolation. We also discussed their active role in pathogenesis. The potential use of exosomes for diagnostic and therapeutic purposes in various diseases is also discussed. Exosomes are perceived to be used as carriers between different locations of body. Exosomes are secreted by all types of cells and are also found abundantly in the body fluids such as: saliva, blood, urine and breast milk. The major role of exosomes is to carry the information by delivering various effectors or signaling molecules between specific cells. This review summarizes current knowledge about the exosomes, their functions, biological and therapeutic use as well as emerging exosomes based therapies that could not be applied before.

Keywords: exosome, microvesicles, microRNA, dendritic cells, drug

Exosomes are the micro-sized vesicles with diameter range of 40–100 nm and have received a lot of attention of scientists over past few decades (1, 2). The word “exosomes” was first used by Rose Johnstone in 1970, who found exosomes from the sheep reticulocytes (3). Laulagnier et al. exfoliated these vesicles from cell lines with ectoenzyme activity (4). Till 1990, these exosomes were considered as best as by-products of cell homeostasis. The revolution in exosomes was observed when it was found that β -cells release functional antigen-transforming exosomes (5).

FORMATION OF EXOSOMES

Exosomes are formed by the endocytic cellular pathway consisting of three different stages: (i) plasma membrane invagination form the endocytic vesicles; (ii) in second stage, inward budding of endosomal membrane starts, which gives rise the multi-vesicular bodies (MVB's); (iii) in third and last stage, MVB's fuse with the plasma membrane and releases the vesicular contents (exosome) (6). The membrane proteins, which undergo the endosomal pathway, exhibit the same stages. Different types of lipidic molecules are known for their involvement in

exosome formation and release like phosphatidic acid and ceramides (7).

Size of the exosomes are dependent on their site of origin as well as lipid bilayer structure in cell (8).

COMPOSITION OF EXOSOMES

Exosomes are unique due to its protein and lipid contents, which provide additional hint for their identification. Exosomes mostly contain fusion proteins and transport proteins (annexins and flotilin), heat shock proteins (HSP) (HSP70), CD's proteins (CD9, CD81), as well as phospholipases and other lipid related proteins (9). All these proteins can be used as positive markers. More than 4400 different proteins can be identified in association with exosomes by mass spectrophotometer and these proteins serve as cargo for intracellular communication (10). Along with proteins, exosomes are also enriched with lipids like cholesterol, sphingolipids, phosphoglycerides, ceramides and short and long saturated fatty acid chains (11). Research indicates that exosomes serve to deliver the prostaglandins to the target cells (9). It has also been investigated that exosomes (Fig. 2) have saccharide

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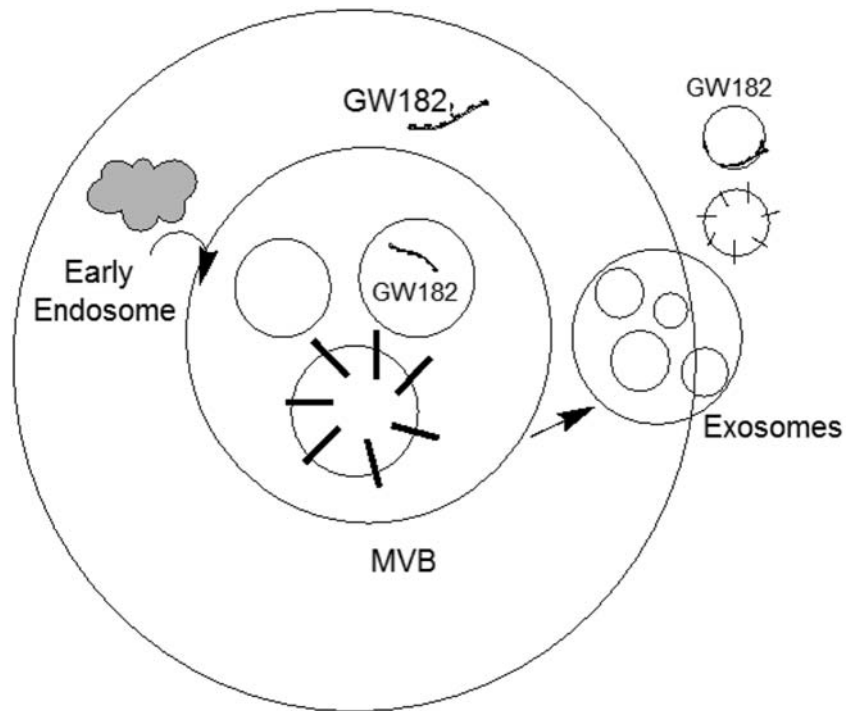


Figure 1. Schematic diagram of exosome production (6)

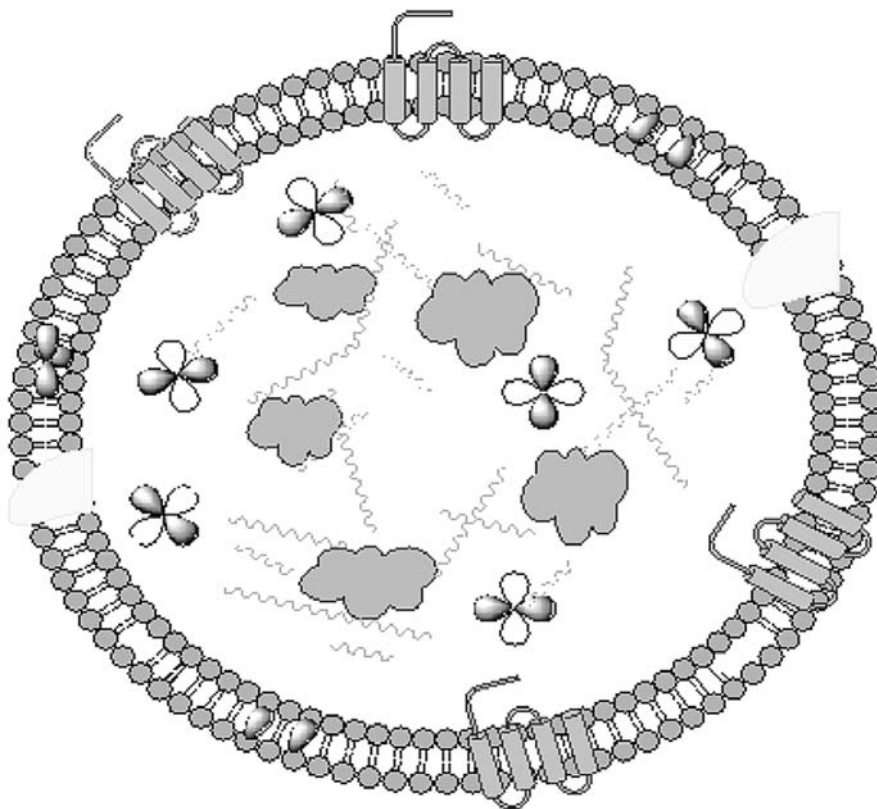


Figure 2. Representation of mid-size exosome (blobs = proteins, ribbon = RNAs) (12)

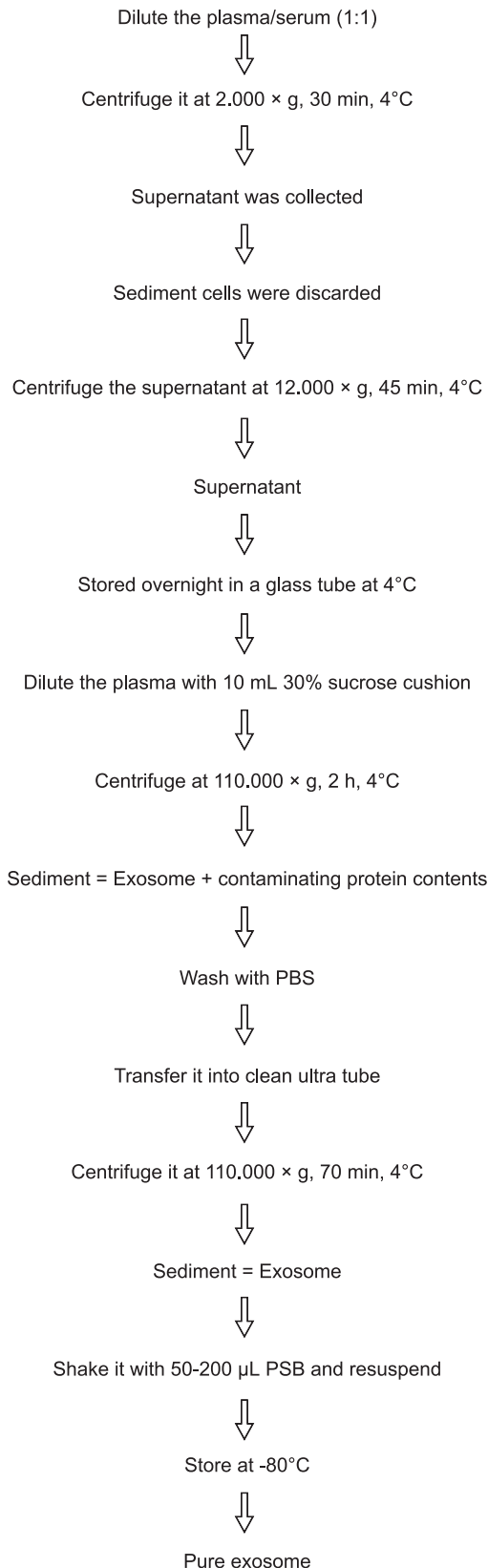


Figure 3. Isolation of exosome by ultra-centrifugation method from plasma/serum (15)

groups in their structure and are enriched with man-nose and glycan (12). It has also been reported that exosomes possess miRNA in a significant amount (13). The cargo function of exosomal RNA is entirely different than that of normal cell RNA (14).

EXOSOMES ISOLATION

Exosomes are isolated by ultracentrifugation method. The exosomes isolation is based on the size of exosomes (15). Exosomes extraction from blood or cell-culture media is complicated because a large number of micro-sized particles are present in media having the same size range as that of exosomes (16). Schematic representation for the isolation of exosome is given in Figure 3.

BIOLOGICAL FUNCTIONS OF EXOSOMES

Multiple cell lines that release the exosomes *in vitro* like neuronal cells, fibroblast cells, adipocytes, intestinal epithelial cells and tumor cell lines have been described. *In vivo*, exosomes are found to be present in many biological fluids like: synovial fluid, breast milk, blood, urine and saliva, amniotic liquid and malignant effusions of ascites. In blood serum, exosomes are almost present in a quantity of 3,000,000 per microliter (17). The first reported biological function of exosomes is as proteins, which are expelled out from reticulocytes during the process of maturation in erythrocytes. Authors believed that particles, which sediment from plasma at 10,000 × g, are circular in nature and name them as exosomes (18). Further research showed that exosome secretion is just like an excretion process to get rid of unnecessary proteins and RNA (4). With the passage of time, more research was being conducted on exosomes discovering that exosomes are found to be secreted by many cell types. On the basis of their origin, exosomes perform a variety of functions. Extensive studies have been done on the facilitator effect of exosomes in immune response (1) and its antigen presenting role has also been extensively reported (19). Exosomal role in coagulation, inflammation and angiogenesis were also reported (20). After the activation, platelets secretes exosomes as well as other shedding microvesicles (21). In this case, exosomes did not perform any role in coagulation. It has been reported that exosomes are involved in dictyostelium cells migration by using chemo-attractant signals (22). Another group of researchers studied the level of miRNA in exosomes of human breast milk for several months during lactation (2). They reported that certain miRNA's like miR-155 and miR-181a, that play

an important role during immune regulation and were present in high concentration during first six months of lactation, were significantly reduced afterward (7). Recent studies demonstrate that the exosomes are not only involved in triggering downstream signaling but they also specifically target the recipient cells and exchange proteins. Exosomes also deliver the specific nucleic acids and work as cargo (23). The unique function of exosomes is cell to cell communication, especially between the far distance cells in the body. Similarly, exosomes play a unique role in spreading various pathogens like virus and prion from one cell to another (24).

EXOSOMES IN DIAGNOSTICS

For last few years, much research has been done on diagnostic aspect of exosomes and it was discovered that almost all the body fluids (blood, saliva, milk, and urine) contained exosomes. Because of unique structure of the exosomes, which possess proteins, lipids and RNAs, it may be useful for the diagnostic purposes (25). In late 1970s, microvesicles (MVs) were derived from the cancer cells in person suffering from Hodgkin's disease (14). Since that day to-date, considerable efforts have been done to use the MVs as diagnostic tool (Table 1). It was reported that MVs levels were elevated in serum, urine and blood in the cancer patient (26). However, microvesicular components may provide important information regarding a disease. For example, mucin bearing MVs are used as diagnostic marker for the diagnosis of adenocarcinoma (27). A proteomic investigation of urine identified eight proteins, which act as an important diagnostic tool in bladder cancer

(28). Thus it can be said that protein portion of the exosomes are the useful tool for the diagnosis of the diseases. In addition, recent studies have showed that cancer patients exhibit different patterns of RNA and miRNA. In cancer patients, RNA and miRNA have been found in circulating MVs form (9).

The PCR of miRNA is a sensitive and stable method for the diagnosis and detection of miRNA in patients' serum, which is a new promising approach to detect disease in early stages. Down-regulation of miR-92a in plasma is the biomarker of hepatocellular carcinoma and leukemia (12).

EXOSOMES AS TARGETED DRUG DELIVERY VEHICLES

Exosomes can be used as targeted drug delivery systems. Alvarez et al. first of all presented and proved this hypothesis (36) by using immature dendritic cells (DCs). They used DCs derived from the bone marrow of mouse as a source of exosomes and these exosomes were devoted as stimulatory molecules such as MHCII and CD80. They purified the exosomes by ultracentrifugation method and used as cargo for siRNA delivery both in *in vitro* and *in vivo* studies. They selected brain as a target tissue in body, because it is believed that blood brain barrier (BBB) is the major obstacle in drug delivery to central nervous system. Sealed functions of BBB are due to the capillary endothelial cells that are tightly sealed by junctions and regulate the barrier functions (19). For ensuring targeted delivery of exosomes, Ratajczak et al. (27) used the novel strategy by utilizing LAMP2B, an exosomal surface protein, that display the targeted peptide on its surface.

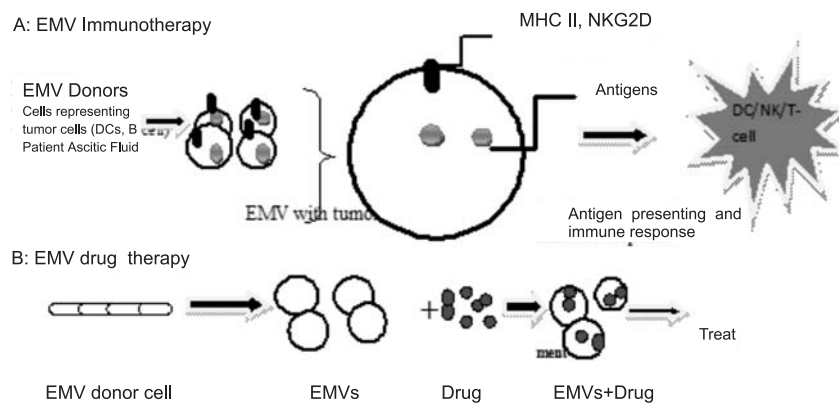


Figure 4. Extracellular membrane vesicle therapy (EMVs) **A:** EMV immunotherapy. Tumor antigen on the membrane surface from different sources was introduced *in vivo* to elicit targeted immune responses. **B:** EMV drug therapy. Drug packaged into/onto EMVs isolated from donor cells to minimize degradation and increase delivery to intended sites (18)

Table 1. Circulating exosomes as potential diagnostic markers for various diseases.

	Sample type	Marker	Disease	Ref.
Quantity	Plasma	PMPs level	Gastric cancer	(29)
	Serum	PMPs level	Prostate cancer	(30)
Protein expression	Ascites	CD24, EpCAM	Ovarian cancer	(31)
	Serum	Tissue factor	General cancer	(32)
	Plasma	Tissue factor	Breast cancer	(33)
	Pleural effusion	SNX25, BTG1	Mesothelioma	(34)
	Urine	Fetuin-A	Acute kidney injury	(9)
miRNA or mRNA expression	Serum	Glioblastoma	Glioblastoma	(10)
	Serum	MAGE-1, HER-2	Gastric cancer	(35)

TREATMENT OF BRAIN INFLAMMATORY DISEASE BY EXOSOMES ENCAPSULATED WITH DRUGS

Zhuang et al. used the encapsulated curcumin (Exo-cur) or JS1124 (Exo-JS1124) inhibitor of signal transducer and activator of transcription and delivered it into the microglia cells through intranasal route. They used lipopolysaccharide (LPS)-induced inflammatory model for the experimental mice to induce inflammation. They showed that mice treated with Exo-cur and Exo-JS1124 were protected from LPS-induced inflammation. They believed that exosomes were selectively taken by the microglia cells and subsequently induced the apoptosis of the microglia cells after its intranasal delivery (30).

EXOSOMES AS AN APPROACH FOR TREATING ARTHRITIS

Dendritic cells (DCs) and T-cells have been used for delivery of immunosuppressive cytokines for the treatment of various collagen induced inflammations in different mouse model (31). DCs are the antigen presenting cells that regulate the immune activity. Various factors are involved in stimulating or suppressing immune responses of DC. DCs have low level of MHC and other molecules such as ICAM-1, so they can suppress T-cell immune response. The immunosuppressive ability of DCs enhanced its genetic modification and genetically modified DCs showed dramatic control in the progression of autoimmune diseases like diabetes and arthritis (24). DCs with viral vectors expressing the immunosuppressive agents exert their effect more pronouncedly than T-cells or fibroblasts (8).

Due to the ability of genetic modification of DCs, they produce distal therapeutic effects specially when exosomes were delivered along with viral vectors (6). Immunosuppressive DCs-exosomes can modify the endogenous immune cells, such as APCs, so they may be responsible for anti-inflammatory effects (1).

EXOSOMES IN IMMUNOTHERAPY AND NERVOUS SYSTEM

Exosomes/MVs (EMVs) have cell to cell communication function for transfer of genetic material (4). The dramatic progress in the research of MVs for drug delivery is due to its low immunogenicity and unique delivering properties. With the help of genetic engineering, EMVs are used to transport the therapeutic drugs either by direct insertion or by loading onto the targeted gene (38). Exosomes also serve as an excellent therapeutic cargo due to its protection rendered to enclosed contents. Due to these possible advantages, EMVs mediated therapy is actively studied and is used in three different fields i.e., immunotherapy, RNA-interference (Fig. 4) and drug delivery (18). The most wide investigational portfolio of EMVs is in immunotherapy, which is an efficient way of cancer treatment through the preparation of vaccines containing antigen presenting cells to recognizes the tumor cells (20). It has been shown that B-lymphocytes secrete EMVs and these EMVs contain MHC-II, which can induces the cell response *in vitro* (19). These EMVs may be used as an emerging therapy in the treatment of various nervous system diseases. Sun et al. developed drug-loaded exosomes (35). They successfully loaded curcumin into EMVs, which significantly reduced the LPS-induced inflammation.

Table 2. Studies on the immunogenicity of tumor-derived exosomes and their vaccines.

Exosome source	Modification	Model	Results	Ref.
Mouse colon carcinoma and melanoma	Parent cells were heat treated	Mouse	Elevated level of Hsp-70, elicit Th 1 response	(1)
Mouse lung carcinoma	Parent cells were heat treated	Mouse	Activates DCs and T-cells and produce immune response	(32)
Human renal cancer	Parent cells were modified to release GPI-IL-12	<i>In vitro</i>	IL-12 promotes the release of IFN- α	(19)
Ascites from colorectal cancer	Exosomes were purified	Phase 1 clinical trials	GM-CSF induce beneficial tumor specific CTL response	(3)

ROLE OF EXOSOMES IN SENESCENCE AND AGING

Senescence is the cellular part of aging of tissues due to the irreversible growth arrest and other physiological changes occurring in cell morphology, cell behavior and function. The miRNAs are small non-coded RNAs, which regulate the gene expression and play an important role in biological processes. Recent developments have shown that exosomes contain miRNAs, which are released into variety of cells and play an important role in cell-to-cell communication and information transfer. Exosomes with miRNAs formed the complex cellular network senescence and contribute to aging (39).

ANTI-TUMORIGENIC ROLE OF TUMOR-DERIVED EXOSOMES

The protein portion of the exosomes reflects the cell type specificity for their cell of origin from which they are secreted. Particularly exosomes, which are derived from tumor may contain tumor-specific antigens on their surface as present in tumor cells (21). Tumor antigens such as carcinoembryonic antigen (CEA) (12) and mesothelin (13) are observed in tumor-derived exosomes. Due to this observation, it is suggested that tumor exosome-based cancer vaccines may be developed. Tumor-derived exosomes might be used as a tumor antigen source, which might be able to induce the CD8⁺ T-cell dependent anti-tumor effects in mice (39). Recently, it was reported that dendritic cells loaded with tumor exosomes elicited the CD8⁺ T-cell response against the tumor cells in malignant gliomas patient (40). For augmenting anti-tumor activity/immunity, tumor derived exosomes have been investigated for direct application (Table 2). Research showed that tumor-derived exosomes produced specific antitumor activity when its parent

cells were genetically modified. These genetically modified exosomes can express pro-inflammatory cytokines such as IL-2 (6). Heat shocked lymphoma cells, which releases the exosomes expressing MHC and other co-stimulatory molecules, induce efficient anti-tumor T cell immunity (40).

CONCLUSION

Increasing research efforts are being done on the exosomes from which we are gaining knowledge on the mechanism of their formation, secretion, *in vivo* pathways, and biological role of their nucleic acid, protein and lipid. With the emergence of exosomes/EMVs responsible for cell to cell communication, researchers gathered the information on their role, both on physiological and pathological functions as well as their use in different therapies. The most interesting aspect of exosomes is their use as vesicular carriers. They carry the large sized molecules such as RNA and proteins that influence gene expression. These microvesicles are similar to viruses and are capable of communication from one cell to another and easily pass the contents of cells across the cell membrane and deliver the macromolecules that are biologically active. Much research had been done on therapeutic applications of the exosomes. Further developments are aimed to ensure therapeutic functions and clinical potential of exosomes including their cargo property, targeting function and different sources of exosomes that enable tissue targeted applications of exosomes.

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ANALYSIS

**NEW RENIN INHIBITORS – STABILITY AND ACTIVITY DETERMINATION.
PART I**DOROTA MARSZALEK^{1*}, ANNA GOLDNIK¹, ALEKSANDER P. MAZUREK², IWONA WINIECKA¹
and PAWEŁ JAWORSKI¹¹Department of Drug Chemistry, Medical University of Warsaw, 1 Banacha St., 02-097 Warszawa, Poland²National Medicines Institute, 30/34 Chełmska St., 00-725 Warszawa, Poland

Abstract: A series of new six potential renin inhibitors containing pseudodipeptides were synthesized. Stability for all compounds (**1-6**) in homogenates of liver, kidney, lung and in serum, gastric, intestinal juice and in the presence of α -chymotrypsin was determined. Compound **5** was unstable, compound **6** was stable, other compounds were partly unstable, compound **2** was stable except kidney homogenate and compound **4** was stable except liver homogenate. Inhibitory activity of the compounds was measured *in vitro* by HPLC determination of lowering concentration of substrate (angiotensinogen) in the presence of renin and the potential renin inhibitor (compounds **1-6**). Compound **2**, **4** and **6** showed inhibitory activity (1.4×10^{-6} , 5.2×10^{-6} , 1.5×10^{-7} M, respectively). Other compounds (**1**, **3**, **5**) showed no inhibitory activity up to 10^{-5} M.

Keywords: HPLC, activity of potential renin inhibitors, renin inhibitors

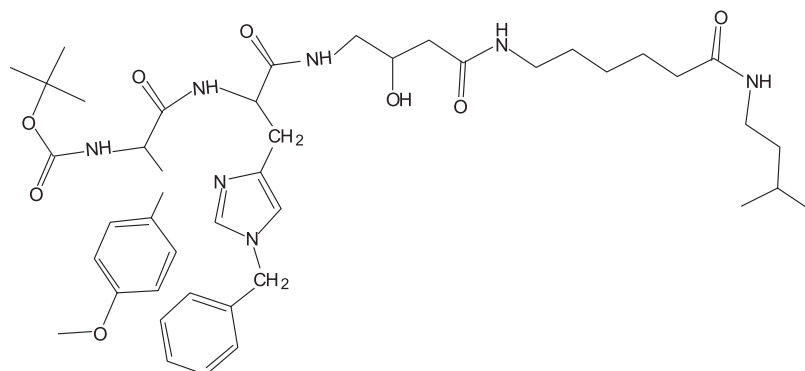
Renin, an aspartic proteinase, catalyzes a specific hydrolysis of the angiotensinogen to give the decapeptide angiotensin I. Angiotensin converting enzyme (ACE), converts it to the octapeptide angiotensin II, which is a very strong vasoconstrictor and it also stimulates aldosterone release and sodium retention. Renin is a specific enzyme that displays specificity for its only one known natural substrate – angiotensinogen. Therefore, the inhibition of renin, which action initiates the renin-angiotensin cascade, has been a highly attractive biological target for new antihypertensive drugs. Drugs that inhibit the renin-angiotensin system, like ACE inhibitors and angiotensin II receptor blockers, are very effective in hypertension treatment but these drugs are characterized by many side effects (they stimulate compensatory mechanism, which results in an increase of angiotensin II level). Therefore, the idea to treat hypertension through the renin inhibition has led to development of many potent renin inhibitors based on the peptide sequence of natural substrate – angiotensinogen.

Many trials to developed effective direct renin inhibitors were not successful (synthesized compounds, which were peptide substrate analogues, were not stable, they revealed low potency or poor

pharmacological profiles). To avoid such problems, new substrates analogous to non-peptic amino acids, peptide-like inhibitors and fully nonpeptic inhibitors were developed (1, 2). Aliskiren is the first renin inhibitor registered at the FDA (3). The structure of aliskiren differed in 8–13 amino acids fragment from the structure of natural substrate – angiotensinogen. It shows high effectiveness and good pharmacokinetic profile.

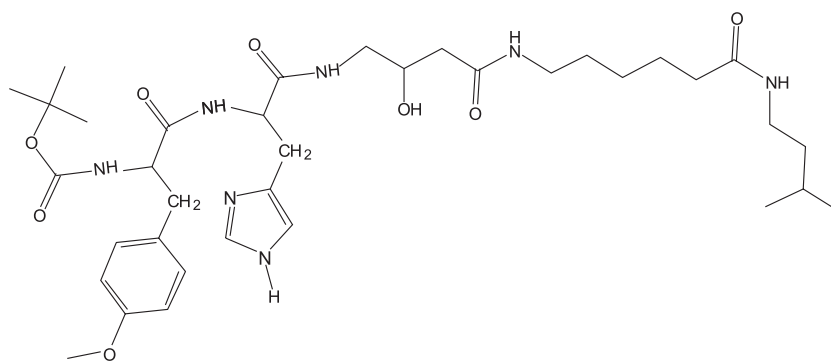
Searching for new renin inhibitors, a series of dipeptide analogues of angiotensinogen have been prepared and they were all derived from renin substrate by replacing the scissile amide bond with a transition-state mimic structure and by incorporating bioisosteric replacements for the Val-10 amide bond. These derivatives showed high inhibiting activity (10^{-6} – 10^{-9} M) (4). Other transition-state renin inhibitors containing the dipeptide transition state mimic structure: (2*S*,4*S*,5*S*)-5-amino-4-hydroxy-2-isopropyl-7-methyloctanoic acid (Leu $\overline{\text{OH}}$ Val) and (2*S*,4*S*,5*S*)-5-amino-4-hydroxy-2-isopropyl-6-cyclohexylhexanoic acid (Cha $\overline{\text{OH}}$ Val) were synthesized (5, 6). The goal of such investigation was to lower the molecular weight, to minimize the number of peptide amide bonds and to enhance *in vivo* stability. All derivatives showed high activi-

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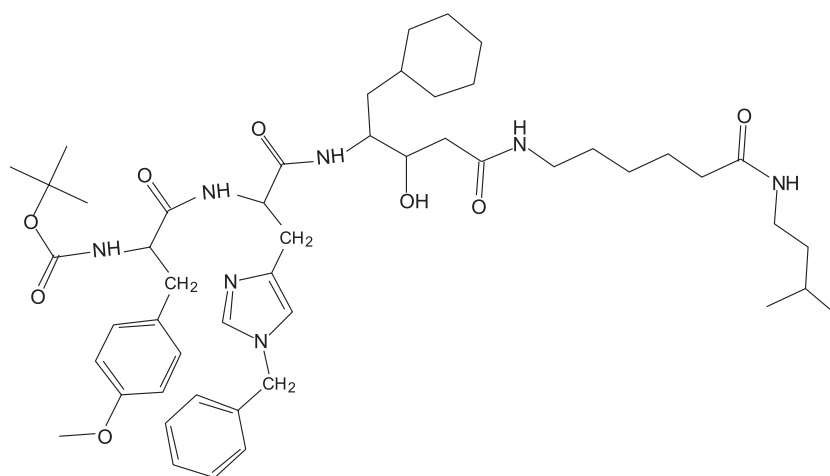
Boc – Phe (4-OMe) – His (N^{im}Bzl) – AHBA – εAhx – Iaa

Compound 1 – [N-(*t*-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}benzylhistidyl-(3*S*,4*S*)-4-amino-3-hydroxybutanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His – AHBA – εAhx – Iaa

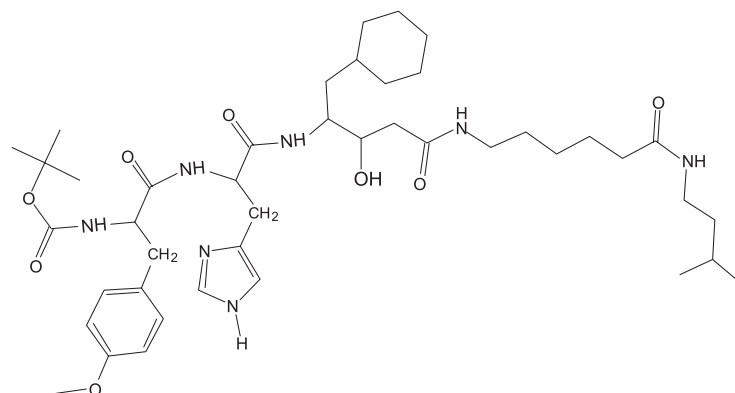
Compound 2 – [N-(*t*-butoxycarbonyl)-4-methoxyphenylalanyl]-(3*S*,4*S*)-4-amino-3-hydroxybutanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His (N^{im}Bzl) – ACHPA – εAhx – Iaa

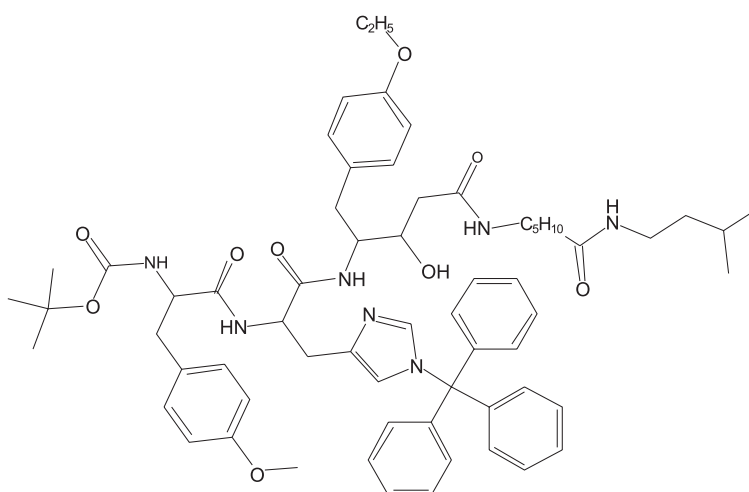
Compound 3 – [N-(*t*-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}benzylhistidyl-(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide

Figure 1. Chemical structures of 6 new renin inhibitors



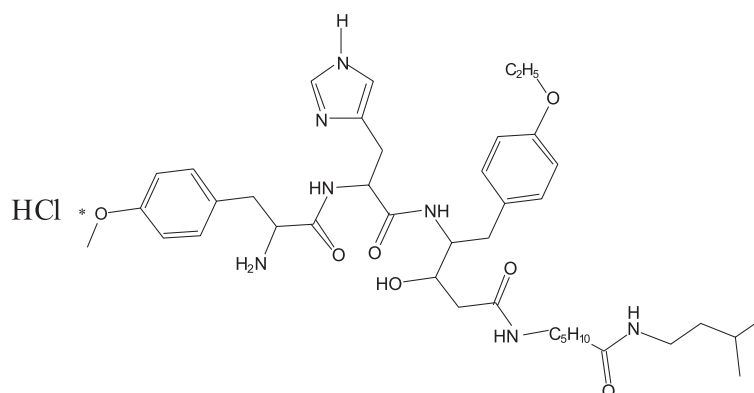
Boc – Phe (4OMe) – His – ACHPA – εAhx – Iaa

Compound 4 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl-(3S, 4S)-4-amino-5-cyclohexyl-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide



Boc – Phe (4-OMe) – His (N^{im}trit) – AEPHPA- εAhx-Iaa

Compound 5 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{im}tritylhistidyl-(3S, 4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide



Phe (4-OMe) – His – AEPHPA- Ahx-Iaa · HCl

Compound 6 – 4-methoxyphenylalanyl-histidyl-(3S, 4S)-4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoyl-ε-aminohexanoic acid isoamylamide hydrochloride

Figure 1. cont

ty (10^{-9} M). Other report presented peptide backbone modifications that lead to greater resistance of the resulting peptides towards enzymatic degradation and some were potent inhibitors of human renin (e.g., Boc-Pro-Phe- N^{α} -MeHis-Leu Ψ -(CHOHCH₂)-Val-Ile-Amp showed inhibiting activity – 2.6×10^{-10} M (7, 8). The synthesis of renin inhibitors (angiotensinogen analogues) having dehydrostatine, Leu Ψ [CH₂S]Val, or Leu Ψ [CH₂SO]Val at P₁-P₁' cleavage site was described (9). Activity of those compounds was between 10^{-6} to 10^{-8} M. Bock et al. obtained a series of statine containing tetrapeptides, modified at the C-terminal with various hydrophobic aromatic groups (10). The inhibiting activity of obtained compounds were between 10^{-6} to 10^{-8} M, e.g., for compound Boc-Phe-His-Sta-Leu-p-

chlorobenzylamide it was 8.1×10^{-8} M. Other peptatin analogues of general formula A-X-Y-Sta-Ala-Sta-R were synthesized (11). Various changes of A, X and Y groups were undertaken to improve the inhibitory activity against human plasma renin. The *tert*-butyloxycarbonyl group and the isovaleryl group were the most effective acyl groups (A). The replacement of Val with Phe residue (X) and incorporation of His or other amino acid with an aliphatic side chain in the position Y lead to an increase of inhibition activity against human plasma renin (10^{-8} M). Changes on C-terminal statine, like estrification or amidification, had no influence on inhibitory potency. Jones et al. (12) used solid-phase method to synthesize a series of peptides (analogues of angiotensinogen), in which statine or new analogues

Table 1. Characterization of new synthesized renin inhibitors.

No.	Compound	Formula	M _r
1	Boc-Phe(4-OMe)-His(BZL)-AHBA-Ahx-Iaa	C ₄₃ H ₆₃ O ₈ N ₇	806.04
2	Boc-Phe(4-OMe)-His-AHBA-Ahx-Iaa	C ₃₆ H ₃₇ O ₈ N ₇	715.92
3	Boc-Phe(4-OMe)-His(BZL)-ACHPA-Ahx-Iaa	C ₅₀ H ₇₅ O ₈ N ₇	902.20
4	Boc-Phe(4-OMe)-His-ACHPA-Ahx-Iaa	C ₄₃ H ₆₉ O ₈ N ₇	812.07
5	Boc-Phe(4-OMe)-His(trit)-AEPHPA-Ahx-Iaa	C ₆₄ H ₈₁ N ₇ O ₉	1092.40
6	Phe-(4-OMe)-His-AEPHPA-Ahx-Iaa × HCl	C ₄₀ H ₅₉ N ₇ O ₇ Cl	784.94

Boc – *tert*-butoxycarbonyl; Iaa – isoamylamide; Ahx – 6-aminoheptanoic acid; AHBA – 4-amino-3-hydroxybutanoic acid; ACHPA – 4-amino-5-cyclohexyl-3-hydroxypentanoic acid; Phe(4-OMe) – 4-methoxyphenylalanine; AEPHPA – 4-amino-5-(4-ethoxyphenyl)-3-hydroxypentanoic acid.

Table 2. Chromatographic and validation parameters.

Compd. no.	Column	Mobile phase (v/v/v)	r	Recovery (%)	CV (%)	Extraction
1	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN-H ₂ O-H ₃ PO ₄ (42 : 58 : 0.1)	0.9976	103.26 ± 8.25	4.93 ± 2.34	alkaline 71%
2	Discovery Wide Pore C ₈ (150 × 4.6 mm)	ACN-H ₂ O-trichloroacetic acid (10 : 90 : 0.1)	0.9988	96.08 ± 10.59	5.13 ± 1.05	acidic 53%
3	Beckman Ultrasphere Octyl (150 × 4.6 mm)	MeOH-H ₂ O-H ₃ PO ₄ (45 : 55 : 0.1)	0.9993	98.99 ± 3.31	5.66 ± 1.28	acidic 63%
4	Symmetry C ₁₈ (150 × 4.6 mm)	ACN-H ₂ O-H ₃ PO ₄ (30 : 70 : 0.1)	0.9972	98.18 ± 6.00	4.48 ± 3.38	acidic 70%
5	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN-H ₂ O-H ₃ PO ₄ (70 : 35 : 0.1)	0.9996	103.21 ± 7.01	10.48 ± 2.85	acidic 81%
6	Symmetry C ₁₈ (150 × 4.6 mm)	ACN-acetate buffer pH 4.0 (40 : 60)	0.9990	100.80 ± 4.92	7.40 ± 4.28	alkaline 20%

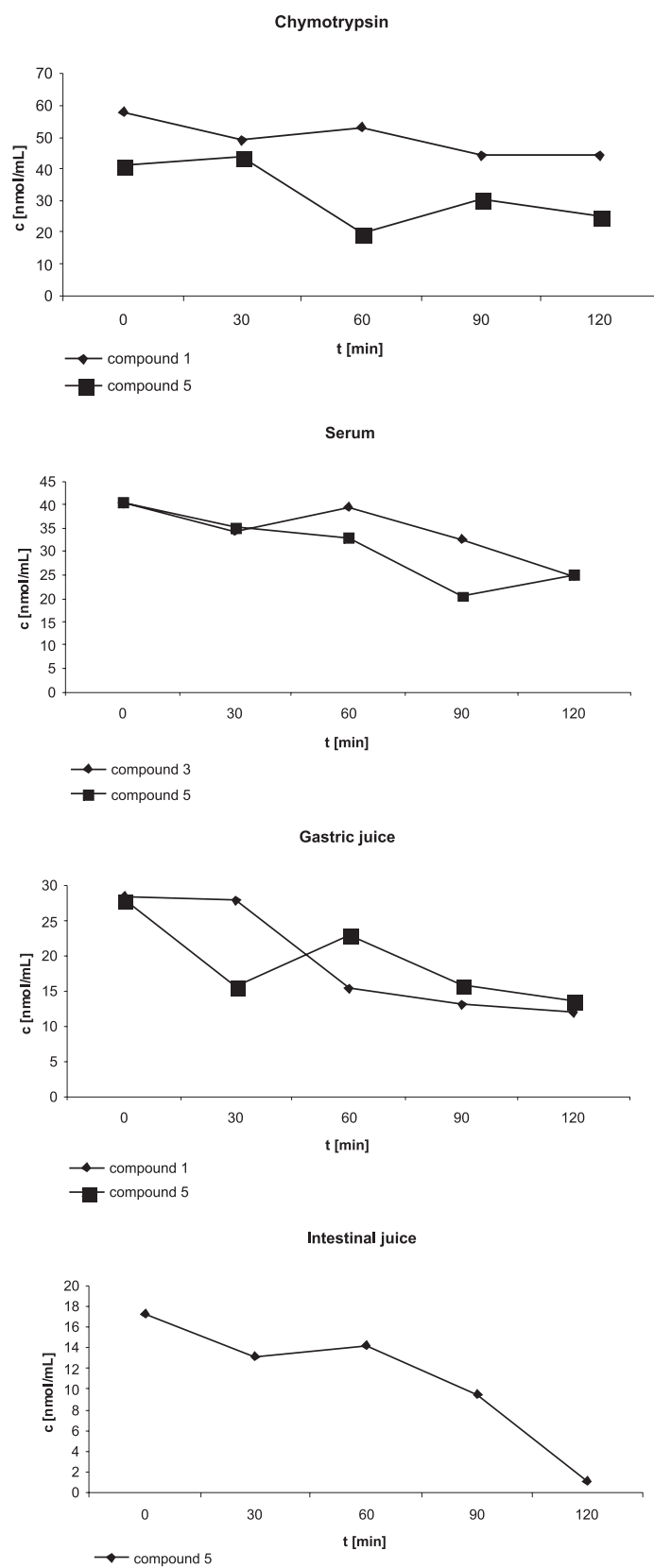


Figure 2. Stability of compounds 1–6 in body fluids and organs (the plots of concentration vs. time)

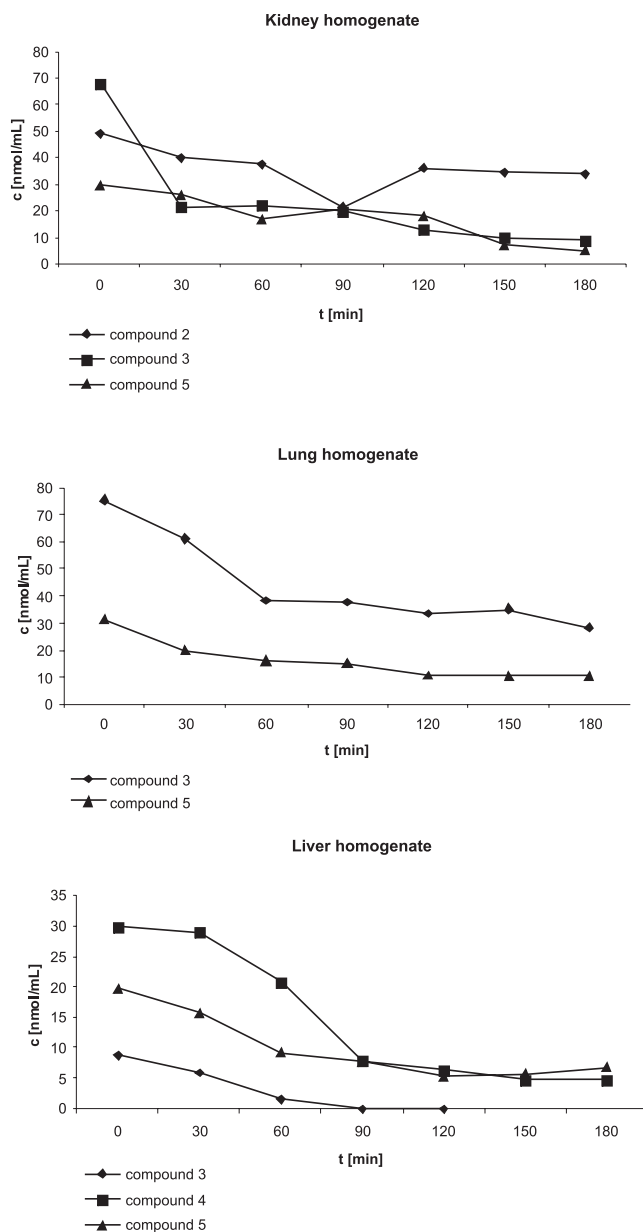


Figure 2. cont.

Table 4. Inhibiting activity of compounds 1-6.

Compd. no.	Human renin IC ₅₀ (M)
1	inactive > 10 ⁻⁵
2	1.4 × 10 ⁻⁶
3	inactive > 10 ⁻⁵
4	5.2 × 10 ⁻⁶
5	inactive > 10 ⁻⁵
6	1.5 × 10 ⁻⁷

10–50 nmol/mL. The wavelength was 213 nm. The columns, mobile phases and validation parameters have been presented in Table 2.

The method of compounds 1–6 determination was validated. Recovery, accuracy and linearity of the analytical procedure are presented in Table 2.

Stock solutions of determined compounds were prepared by dissolving each compound in methanol. The final working concentration for the examined substances was 100 nmol/mL. The liquid-

liquid extraction method was used for all biological material.

Determination of enzymatic stability of compounds 1–6 *in vitro*

The stability of all compounds in body fluids and organ homogenates was examined. The concentrations of compounds were measured at different time points during incubation in biological material. The developed HPLC method was used to determine the concentration of compounds 1–6 isolated from biological material by liquid-liquid extraction with the use of diethyl ether.

Stability determination of compounds 1–6 in liver, kidney and lung enzymes

The homogenates of body organs (40%) in 0.1 mole/L TRIS solution (pH = 8.4) were prepared. Each homogenate was spiked with each of 6 compounds and incubated at 37°C. The samples were collected at the time points 0, 30, 60, 90, 120, 150, 180 min and then isolated from homogenates with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–6 in serum, gastric and intestinal juice

Each compound was dissolved in certain amount of serum or freshly prepared gastric or intestinal juice (according to USP). The solutions were incubated at 37°C and the samples were collected at the time points 0, 30, 60, 90, 120 min. The samples were isolated from body fluids with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Stability determination of compounds 1–6 in the presence of α -chymotrypsin

α -Chymotrypsin was dissolved in phosphate buffer solution pH 7.8. The solution was incubated at 37°C and the samples were collected at the time points 0, 30, 60, 120 min. Then, the samples were isolated from the solution with diethyl ether, evaporated to dryness, dissolved in mobile phase and determined by HPLC. Results are presented in Table 3 and Figure 2.

Determination of inhibition activity of compounds 1–6 vs. human renin

Renin inhibiting activity of the synthesized potential inhibitors was determined *in vitro*. The modified HPLC method of determination of

angiotensinogen (substrate) concentration was used (column: Wild Pore C₈ 15 cm × 4.6 mm, mobile phase MeOH : H₂O : H₃PO₄ 40 : 60 : 0.1 (v/v/v), wavelength 213 nm). The HPLC method was modified in comparison with other method (17), used to determine the renin inhibitor activity. The modification includes change of chromatographic conditions and change of the method of inhibiting activity determination. Instead of assay of concentration of tetrapeptide (which is the product of reaction), the concentration of angiotensinogen (substrate) was measured. To check if modified method was reliable in activity determination, we compared results of activity measurements obtained by the method described in this paper with the results obtained with our modified method for renin inhibitor (Boc-Phe-His-Sta- ϵ Ahx-OMe) (18). The results of inhibiting activity for method described in the paper and modified method were IC₅₀ = 5.0 × 10⁻⁹ M and IC₅₀ = 5.3 × 10⁻⁹ M, respectively (18). These results confirmed that the modified method was reliable.

The inhibition of human renin was determined after its incubation with angiotensinogen and with each of compounds 1–6. Human renin 9 mU.G. was incubated with 6 mM angiotensinogen in 30 mM citrate-phosphate buffer (pH 7.4) for 2 h at 37°C with lowering amount (10⁻⁵, 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹ M) of each renin inhibitor prepared in phosphoric-citric buffer pH 7.4. To stop the reaction after 2 h, the sample was immersed in boiling water for 2 min. To control the activity assay, the sample contained only human renin and angiotensinogen was prepared and the activity was measured in time 0 and after 2 h of incubation.

The renin inhibitory activity was designed in terms of the IC₅₀, which is the molar concentration of the examined inhibitor causing 50% inhibition of the control renin activity.

Results are presented in Table 4.

RESULTS AND DISCUSSION

Determination of enzymatic stability of 6 new compounds have been performed in test *in vitro*. The results showed that compound 6 was stable, compound 5 was totally unstable and all other compounds were partly stable in body fluids and organ homogenates. Compound 2 was unstable in kidney homogenate, compound 4 in liver homogenate, compound 1 in gastric juice and in the presence of α -chymotrypsin and compound 3 in serum, kidney, lung and liver homogenates.

Determination of inhibiting activity of compounds 1–6 against human renin had been performed *in vitro*.

The results showed that the removal of substituents of His was necessary to obtain biologically active compound and made compound more stable. Simultaneous removal of Boc group caused that compound was stable in body fluids and organ homogenates and showed the highest inhibitory activity (1.5×10^{-7} M). Compound with AHBA group showed activity 10^{-6} and was rather stable (decomposed only in kidney homogenate). Compound **4** had four times lower activity than compound **2** and was rather stable (decomposed only in liver homogenate), the half-time was about 60 min. The presence of trityl group caused compound unstable in all homogenates and body fluids. The removal of Boc group increased metabolic stability and increased solubility in water.

The search for other compounds with higher potency is under way.

Acknowledgments

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NEW RENIN INHIBITORS – STABILITY AND ACTIVITY DETERMINATION.
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Abstract: A series of new six pseudodipeptic potential renin inhibitors were synthesized. Enzymatic stability for all compounds (**1–6**) in homogenates (liver, kidney, lung) and body fluids (serum, gastric, intestinal juice) and α -chymotrypsin was determined. Compound **4** was stable, compound **5** was unstable and compounds **1**, **2**, **3**, **6** were partly unstable. Inhibitory activity of the compounds was measured *in vitro* by HPLC determination of lowering concentration of substrate (angiotensinogen) in the presence of renin and the potential renin inhibitor (compounds **1–6**). Compound **4**, **5**, **6** showed inhibitory activity (0.9×10^{-6} , 1.3×10^{-5} , 2.2×10^{-6} M, respectively). Other compounds showed no inhibitory activity up to 10^{-5} M.

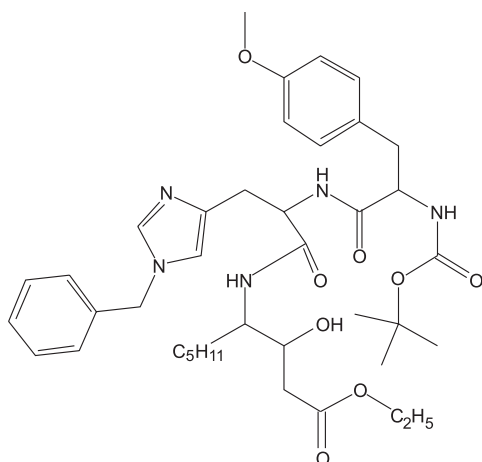
Keywords: HPLC, inhibition of renin activity, renin inhibitors

Hypertension is a major risk for cardiovascular and kidney diseases. Many of patients with hypertension, using antihypertensive drugs, do not have their blood pressure controlled to recommended target levels (< 140/90). Therefore, the renin-angiotensin system has been a highly attractive biological target for new drugs, which could regulate the blood pressure. Inhibition of angiotensin converting enzyme (ACE) has led to development of effective antihypertensive drugs. The other possibility to interrupt of renin-angiotensin system is to inhibit renin. Renin, an aspartyl protease, is involved in the first step of enzymatic cascade. Numerous laboratories have tried to develop clinically effective direct renin inhibitors of high potency and stability (**1–11**). The purpose of these trials was to search for new active inhibitors with simple structure, good bioavailability and easy synthesis. The first in a new class of oral, nonpeptide direct renin inhibitor for treatment of hypertension is aliskiren. The structure of aliskiren differs from the structure of 8–13 human angiotensinogen fragment. Searching for a new inhibitors, a series of compounds with dipeptide replacement [(3*S*,4*S*)-4-amino-5-cyclohexyl-3-hydroxypentanoic acid (ACHPA)] in their molecules were synthesized (**12**). Some of them comprised two additional analogs of dipeptide: (3*S*,4*S*)-4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) and (3*S*,4*S*)-4-amino-3-hydroxy-6-methylheptanoic acid (statine). The inhibitory activity was low, so the authors conclude that renin inhibitors

ought to have one or two, but not three dipeptide analogs in the molecule. Bock et al. synthesized a series of statine-containing tetrapeptides, modified at the carboxy terminus with hydrophobic aromatic groups (**13**). The Phe amide group, known to be a site of hepatic metabolism, was replaced with nonpeptidic groups. It caused the reduction of peptide chain length but also lowering of activity. Searching for more active renin inhibitors the pentapeptide BOC-Phe-Phe-difluorostatone-Leu-Phe-NH₂ was synthesized (**14**). This compound contains a difluoromethylene ketone group. It showed an increase in inhibiting activity in animal models.

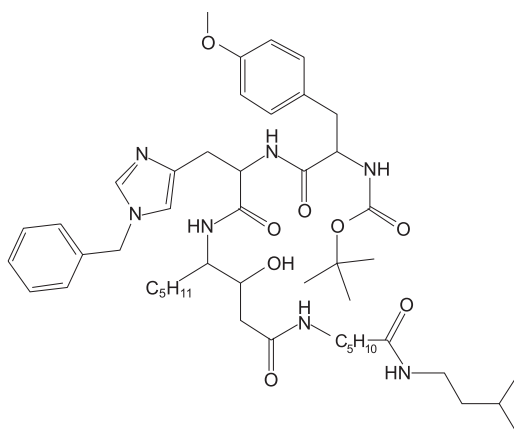
In our previous paper, we presented a series of new six pseudodipeptic potential renin inhibitors. Enzymatic stability for all compounds (**1–6**) in homogenates (liver, kidney, lung) and body fluids (serum, gastric, intestinal juice) and α -chymotrypsin was determined. Inhibitory activity of the compounds was measured *in vitro* by HPLC determination of lowering concentration of substrate (angiotensinogen) in the presence of renin and the potential renin inhibitor (compounds **1–6**). Compound **2** and **4** showed inhibitory activity 1.4×10^{-6} and 0.9×10^{-6} M, respectively, and compound **6** – 1.5×10^{-7} M. Other compounds showed no inhibitory activity up to 10^{-5} M. To continue search for the effective renin inhibitors, 6 new modified compounds of potentially inhibiting activity were synthesized. The chemical structures of them are presented in Figure 1.

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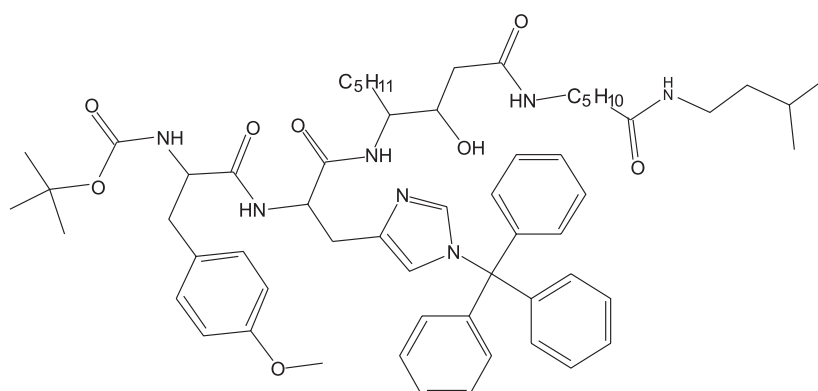
Boc-Phe(4-OMe)-His(N^{imm}BZL)-AHNA-OEt

Compound 1 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{imm}benzylhistidyl-(3S,4S)-4-amino-3-hydroxynonanoyl-Ĺ-aminohexanoic acid ethyl ester



Boc-Phe(4-OMe)-His(N^{imm}BZL)-AHNA-εAhx-Iaa

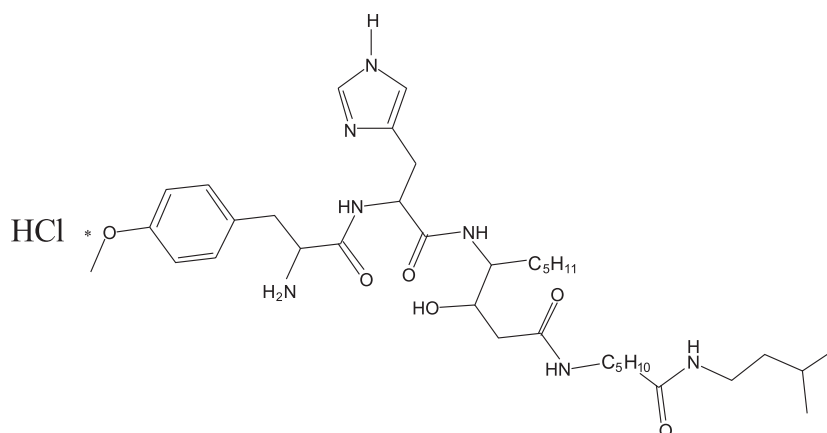
Compound 2 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{imm}benzylhistidyl-(3S,4S)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamylamide



Boc-Phe(4-OMe)-His(N^{imm}trityl)-AHNA-εAhx-Iaa

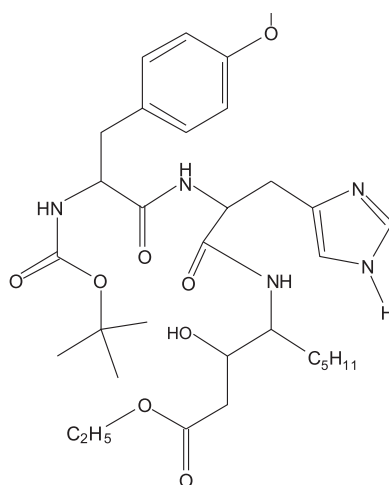
Compound 3 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-N^{imm}tritylhistidyl-(3S, 4S)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamylamide

Figure 1. Chemical structure of 6 new renin inhibitors



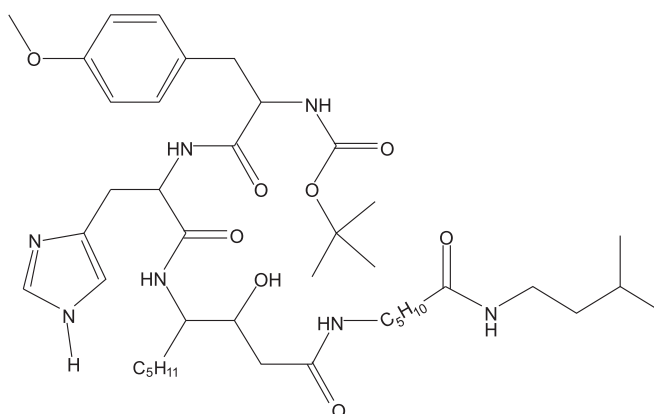
Phe(4-OMe)-His-AHNA-εAhx-Iaa × HCl

Compound 4 – 4-methoxyphenylalanyl-histidyl-(3S,4S)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamylamide hydrochloride



Boc-Phe(4-OMe)-His-AHNA-OEt

Compound 5 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-(3S,4S)-4-amino-3-hydroxynanoylethyl ester



Boc-Phe(4-OMe)-His-AHNA-εAhx-Iaa

Compound 6 – [N-(t-butoxycarbonyl)-4-methoxyphenylalanyl]-(3S,4S)-4-amino-3-hydroxynonanoyl-ε-aminohexanoic acid isoamylamide

Figure 1. cont

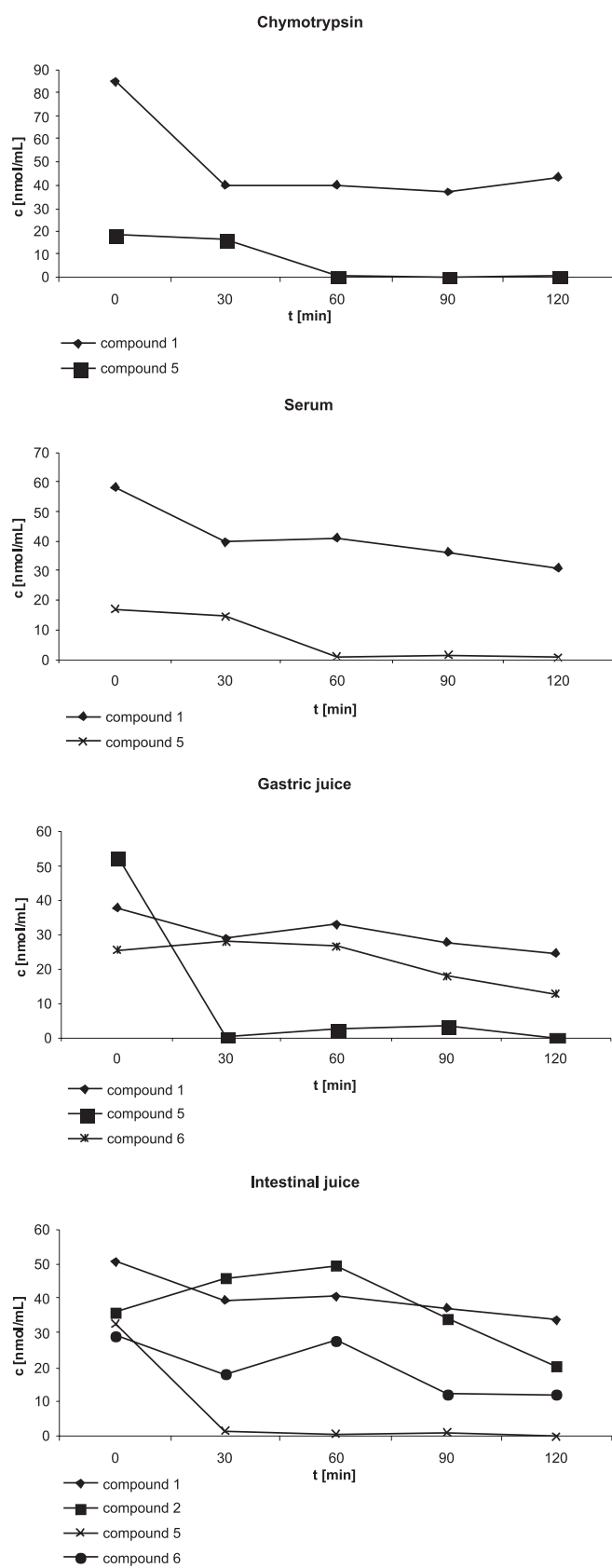


Figure 2. Stability of compound 1-6 in body fluids and organs (the plots concentration vs. time)

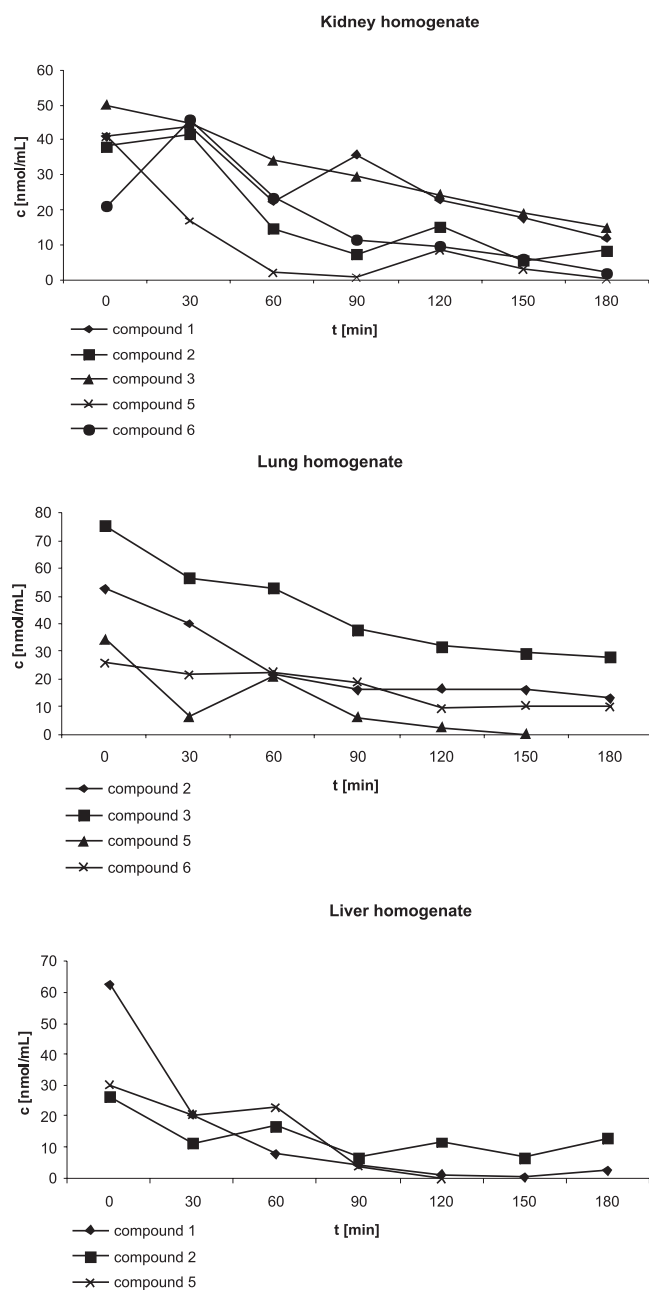


Figure 2. cont.

The purpose of this study was to determine the stability of the new synthesized compounds in homogenates of body organs and body fluids (*in vitro*) and to check the inhibiting activity of six potential renin inhibitors (*in vitro*).

EXPERIMENTAL

Materials and reagents

New renin inhibitors synthesized in the

Department of Drug Chemistry, Medical University of Warsaw are presented in Table 1. Chemical structures are shown in Figure 1.

Renin human and α -chymotrypsin from bovine pancreas were purchased from Sigma. Angiotensinogen was purchased from Bachem.

Apparatus and methods

The details are the same as in the preceding paper.

Table 1. Characterization of new synthesized renin inhibitors.

Compd. no.	Compound	Formula	M _r
1	Boc-Phe(4-OMe)-His(BZL)-AHNA-OEt	C ₃₈ H ₅₅ N ₃ O ₇	693.89
2	Boc-Phe(4-OMe)-His(BZL)-AHNA-Ahx-Iaa	C ₄₇ H ₇₁ N ₇ O ₈	862.12
3	Boc-Phe(4-OMe)-His(trit)-AHNA-Ahx-Iaa	C ₆₀ H ₈₁ N ₇ O ₈	1028.36
4	Phe-(4-OMe)-His-AHNA-Ahx-Iaa · HCl	C ₃₅ H ₅₈ N ₇ O ₆ Cl	708.36
5	Boc-Phe(4-OMe)-His-AHNA-OEt	C ₃₁ H ₄₉ O ₇ N ₅	603.761
6	Boc-Phe(4-OMe)-His-AHNA-Ahx-Iaa	C ₄₀ H ₆₅ O ₈ N ₇	772.001

Boc – *tert*-butoxycarbonyl, Iaa – isoamylamide, Ahx – 6-aminohexanoic acid, AHNA – 4-amino-3-hydroxynonanoic acid, Phe(4-OMe) – 4-methoxyphenylalanine

Table 2. Parameters of chromatographic determination and validation procedure of determined compounds.

Compd. no.	Column	Mobile phase (v/v/v)	r	Recovery (%)	CV (%)	Extraction
1	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN–H ₂ O–H ₃ PO ₄ (42 : 58 : 0.1)	0.9988	103.14 ± 8.12	4.96 ± 2.37	alkaline 71%
2	Beckman Ultrasphere Octyl (150 × 4.6 mm)	MeOH–H ₂ O–H ₃ PO ₄ (65 : 35 : 0.1)	0.9926	100.36 ± 5.03	6.8 ± 1.64	alkaline 80%
3	Discovery Wide Pore C ₈ (150 × 4.6 mm)	MeOH–H ₂ O–H ₃ PO ₄ (40 : 60 : 0.1)	0.9999	100.13 ± 1.87	5.11 ± 1.65	acidic 67%
4	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN–acetate buffer pH 4.0 (40 : 60)	0.9932	101.99 ± 9.05	5.82 ± 1.65	acidic 67%
5	Symmetry C ₁₈ (150 × 4.6 mm)	ACN–H ₂ O–H ₃ PO ₄ (80 : 20 : 0.1)	0.9908	100.84 ± 5.95	4.06 ± 2.95	acidic 64%
6	Beckman Ultrasphere Octyl (150 × 4.6 mm)	ACN–H ₂ O–H ₃ PO ₄ (80 : 20 : 0.1)	0.9940	98.17 ± 6.00	6.83 ± 2.83	alkaline 47%

Table 3. Stability of compounds 1–6.

Compd. no.	α-Chymotrypsin	Serum	Gastric juice	Intestinal juice	Kidney homogenate	Lung homogenate	Liver homogenate
1	unstable	first-order reaction t _{0.5} = 157 min	unstable	first-order reaction t _{0.5} = 239 min	unstable	stable	unstable
2	stable	stable	stable	unstable	unstable	first-order reaction t _{0.5} = 92 min	unstable
3	stable	stable	stable	stable	first-order reaction t _{0.5} = 103 min	first-order reaction t _{0.5} = 122 min	no results*
4	stable	stable	stable	stable	stable	stable	stable
5	unstable	first-order reaction t _{0.5} = 25.7 min	unstable	unstable	unstable	unstable	unstable
6	stable	stable	unstable	unstable	first-order reaction t _{0.5} = 50 min	first-order reaction t _{0.5} = 111.8 min	stable

* it was not possible to isolate compound 3 from biological matrix due to very strong hydrophobic bonds.

Table 4. Inhibiting activity of compounds 1–6.

Compound no.	Human renin IC ₅₀ (M)
1	inactive > 10 ⁻⁵
2	inactive > 10 ⁻⁵
3	inactive > 10 ⁻⁵
4	0.9 × 10 ⁻⁶
5	1.3 × 10 ⁻⁸
6	2.2 × 10 ⁻⁶

Determination of enzymatic stability of compounds 1–6 *in vitro*

The stability of compounds 1–6 in body fluids and organ homogenates was examined similarly as described in the preceding paper.

Determination of inhibition activity vs. human renin of compounds 1–6

Renin inhibiting activity of the synthesized potential inhibitors 1–6 was determined *in vitro* similarly as described in the preceding paper. The results are presented in Table 4.

RESULTS AND DISCUSSION

Determination of enzymatic stability and determination of inhibiting activity of compounds 1–6 against human renin have been performed in test *in vitro*. According to the results of that investigation it was shown that compound 1, 2 and 3 showed no inhibiting activity. The presence of large trityl substituent of His in the structure of compound 3 caused increased strength of hydrophobic bonds with biological matrix, which made extraction of that compound from liver homogenate impossible. A removal of Boc group of Phe and substituent of His in compound 4 caused that it was stable in all homogenates and body fluids. It is possible to conclude that active renin inhibitors should contain amino acid His without any substituents. Compound 5, which contained ester bond in C-terminus, was totally metabolically unstable but showed the highest activity IC₅₀ = 1.3 × 10⁻⁸ M. A removal of Boc group and conversion of compound 4 to hydrochloride increased twice the inhibiting activity. It could be caused by an increase of solubility. This compound was also more stable. The Ahx-Iaa substituent in C-terminus of compounds 2 and 3 protected C-terminus and increased the metabolic stability but reduced inhibiting activity.

The search for other compounds with expected higher potency will be continued.

Acknowledgments

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DEVELOPMENT STUDIES ON DETERMINATION OF PRESERVATIVES DECOMPOSITION PRODUCTS

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Abstract: Preservatives are chemical substances whose role is to protect medicinal products against harmful changes caused by microorganisms. They are added to sterile medicinal products, such as eye drops and multi-dose solutions for injections, as well as to non-sterile products, such as water oral solutions, creams, gels, suppositories and capsules with liquid content. The most commonly used preservatives include: benzyl alcohol, butyl, ethyl, methyl and propyl p-hydroxybenzoates and their sodium salts. In medicinal products benzyl alcohol slowly oxidizes to benzaldehyde and benzoic acid while esters of p-hydroxybenzoic acid hydrolyze to p-hydroxybenzoic acid. HPLC methods were elaborated for identification and quantitative determination of the parabens, benzyl alcohol, active substances as well as their impurities in pharmaceuticals: oral solutions Ameril and Efforil (contain cetirizine hydrochloride or etilefrine hydrochloride and parabens), eye drops Difadol (contains diclofenac sodium and benzyl alcohol) and cream Tenasil (contains terbinafine hydrochloride and benzyl alcohol). The HPLC systems consisted of columns: Supelcosil LC-DP, Inertsil ODS-3 or Discovery HS F5 and three mobile phases – mixtures of acetonitrile with buffers of various pH (3, 5 and 7) in proportions 45 : 55 (v/v). These systems have been characterized with appropriate selectivity (all the R_s values > 2) and sensitivity (LOD approx. 0.01 µg/mL). They also demonstrated satisfactory precision and a linear dependence between the analyte content and the peak area.

Keywords: benzyl alcohol, esters of p-hydroxybenzoic acid, HPLC

Preservatives are chemical substances whose role is to protect food products, stimulants, medicinal products and cosmetics against harmful changes caused by microorganisms (1). When added in proper concentrations, preservatives inhibit the growth of microorganisms during manufacturing and use of medicinal products (2). They should be tasteless, odorless and colorless. In concentrations used, they should be soluble and non-toxic as well as physiologically and chemically compatible. At present, it is recommended – as more effective – to use mixtures of preservatives.

Preservatives are added to sterile medicinal products, such as eye drops and multi-dose solutions for injections, as well as to non-sterile products, such as water oral solutions, creams, gels, suppositories and capsules with liquid content. It is allowed in Poland to use as preservatives the substances listed in Appendix no. 1 to the Regulation of Health Minister dated 16 January 2003, on preservatives, sweeteners, colors and antioxidants that may be included in medicinal products (Journal of Laws No.

19, 2003, item 169). These compounds comprise phenols, alcohols, organic acids, organic mercuric compounds and quaternary ammonium bases. The most commonly used preservatives include: benzyl alcohol, butyl, ethyl, methyl and propyl p-hydroxybenzoates and their sodium salts. According to the aforementioned Regulation, all preservatives should meet the requirements of the European Pharmacopoeia and their content in medicinal products should be determined quantitatively.

Benzyl alcohol, as a preservative in medicinal products (oral solutions and solutions for injections), is used in concentration up to 2%. Benzyl alcohol reveals incompatibilities with oxidizing agents and strong acids. Its antimicrobial activity is reduced in the presence of non-ionic surfactants, such as, for example, polysorbate 80 (1, 3). In medicinal products it oxidizes slowly to benzaldehyde and benzoic acid when exposed to air (4). Three papers have been found on determination of benzaldehyde contents in injections with diclofenac with the use of polarographic method (5) and in

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Table 1. Times of retention (t_r), resolution between the neighboring peaks (R_s) and peak asymmetry (A10%) of tested preservatives and their decomposition products in chromatographic system acc. to Ph. Eur. monographs for methylparaben. Mobile phase: 0.05 M solution of KH_2PO_4 and methanol 35 : 65 (v/v). Columns used: Zorbax Rx C18 (in accordance with the monograph) and columns selected for tests ODS-3, DP, HS F5.

Substance*	Column Rx C18			Column ODS-3			Column DP			Column HS F5		
	t_r	R_s	A10%	t_r	R_s	A10%	t_r	R_s	A10%	t_r	R_s	A10%
p-HBAC	1.5	–	1.4	2.8–3.2	–	triple peak	3.2–3.3	–	double peak	3.6–4.1	–	triple peak
MP	2.2	2.6	1.9	5.1	7.5	1.2	3.9	1.3	1.3	6.3	7.2	1.1
EP	2.7	2.3	1.7	6.6	5.1	1.2	4.0	0.6	1.2	7.7	5.3	1.2
PP	3.7	3.9	1.5	10.0	7.5	1.1	4.3	0.9	1.1	10.0	7.0	1.1
BP	5.7	5.6	1.3	14.8	14.7	1.0	4.6	1.2	1.1	13.7	6.8	1.1
BA-ol	2.1	1.1	1.1	4.5	2.8	1.2	3.7	1.8	1.1	4.7	–	1.4
BAC	1.9	–	0.8	3.8	–	1.8	3.4	–	1.4	5.6	3.8	1.8
BAId	2.49	2.0	1.5	5.7	4.2	0.9	4.0	1.3	1.8	6.0	1.8	1.1

* abbreviations see text.

injections with diclofenac, piroxicam and vitamin B complex with the use of GC method (6, 7). Benzyl alcohol and diclofenac were determined in various medicinal products with spectrophotometric method (8).

Esters of p-hydroxybenzoic acid (parabens) show desirable preserving action within a quite wide range of pH, from weak-acid to weak-alkaline (pH 4 – 8), though in general, they are the most effective in acid environment. Above pH 8, the effectiveness of its action decreases due to rapid progress of hydrolysis. The parabens are more active against yeast and mould than against bacteria. They are also more active against Gram positive than against Gram negative bacteria. Antibacterial action of individual esters increases together with the increased chain length, but their solubility decreases. This problem may be omitted by the use of more soluble sodium salts of the esters, however, this raises the pH of poorly buffered formulations. Antibacterial activity may also be increased by the use of a mix of esters, for example of methyl and propyl p-hydroxybenzoates.

Antibacterial effect of parabens decreases in the presence of macromolecular substances, such as polysorbate 80, as the micelles that are formed close the esters inside. Addition of propylene glycol (10%) increases the adverse interactions in the presence of non-ionic surfactants. Esters are also incompatible with other substances, such as: bentonite, magnesium trisilicate, talc, sorbitol and atropine (1, 3).

Esters of p-hydroxybenzoic acid are used as preservatives mainly in oral solutions and preparations administered on skin. In the past, these were also used in solutions for injections and in the eye drops, but at now this application is being abandoned because of their irritating effect. Additionally, in case of medicinal products administered on eyes, a significant disadvantage of parabens is their poor impact on Gram-negative bacteria, in particular against *Pseudomonas aeruginosa*. Most commonly, a mixture of two esters methyl and propyl is used at concentrations up to 0.25%. A product of esters hydrolysis, p-hydroxybenzoic acid, shows no antibacterial effect (2).

In the available literature, several studies were found describing determination of various esters of p-hydroxybenzoic acid in medicinal products with micellar electrophoresis (9, 10) and HPLC methods (11, 12). Only one of these papers takes into consideration determining the content of parabens hydrolysis product – p-hydroxybenzoic acid (12). Stability of water solutions of p-hydroxybenzoic

Table 2. Times of retention (t_r), resolution between the neighboring peaks (R_s) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the ODS-3 column (Inertsil ODS-3, 5 μ m, 250 \times 4.6 mm).

Substance*	t_r			R_s			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	3.6	3.3	2.4	–	–	–	1.1	1.2	1.2
MP	5.9	5.9	5.9	11.1	12.1	14.5	1.0	1.1	1.1
EP	7.9	8.4	7.9	7.8	7.7	7.5	1.0	1.0	1.1
PP	11.5	11.3	11.4	10.7	10.3	10.1	1.1	1.0	1.0
BP	17.6	17.3	17.3	12.8	12.3	12.1	1.0	1.0	1.1
BAol	5.1	5.1	5.2	–	4.4	11.4	1.1	1.1	1.1
BAc	5.6	4.2	2.6	2.2	–	–	1.1	1.6	1.3
BAld	8.4	7.9	8.6	11.8	11.2	13.3	1.1	1.0	1.0

* abbreviations see text.

Table 3. Times of retention (t_r), resolution between the neighboring peaks (R_s) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the DP column (Supelcosil LC- DP, 5 μ m, 250 \times 4.6 mm).

Substance*	t_r			R_s			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	3.8	3.5	2.8	–	–	–	1.0	1.3	1.1
MP	4.6	4.5	4.6	1.7	4.3	16.9	1.0	1.2	1.1
EP	5.1	4.9	5.0	7.8	1.7	2.1	1.0	1.3	1.1
PP	5.8	5.4	5.6	3.1	2.2	2.7	1.0	1.3	1.1
BP	6.7	6.1	6.5	3.9	2.7	3.2	1.0	1.3	1.1
BAol	4.4	4.3	4.4	–	2.9	17.9	1.0	1.4	1.1
BAc	4.5	3.7	2.8	1.0	–	–	1.0	1.6	1.1
BAld	5.2	5.0	5.1	3.8	3.1	4.3	1.0	1.2	1.1

* abbreviations see text.

Table 4. Times of retention (t_r), resolution between the neighbouring peaks (R_s) and peak asymmetry (A10%) of tested preservatives and their decomposition products in elaborated chromatographic systems on the HS F5 column (Discovery HS F5, 5 μ m, 250 \times 4.6 mm).

Substance*	t_r			R_s			A10%		
	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7	pH ~3	pH ~5	pH ~7
p-HBAc	4.0	3.9	2.4-2.5	–	–	–	1.2	1.3	double peak
MP	6.0	5.9	6.1	10.6	9.6	32.3	1.0	1.1	1.2
EP	7.3	7.2	7.4	5.9	5.6	5.6	1.0	1.1	1.3
PP	9.3	9.0	9.4	7.8	7.2	7.4	1.0	1.1	1.3
BP	12.2	11.7	12.3	9.4	8.6	8.6	1.0	1.0	1.4
BAol	5.2	5.1	5.1	–	–	26.2	1.0	1.1	1.1
BAc	5.6	6.8	2.9	2.3	4.8	–	1.1	2.7	1.1
BAld	7.8	7.7	7.5	10.6	2.2	9.8	1.0	1.0	1.3

* abbreviations see text.

Table 5. The linear relationship between analyte concentration (c) and peak area (x) for parabens and benzyl alcohol in elaborated chromatographic systems. The coefficients of correlation *r* given in brackets.

Substance*	$c = ax + b$					
	Column ODS-3			Column DP		
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3 p	H~5
MP	1.05E-05x-2.82 (r = 0.9989)	8.63E-06x-2.67 (r = 0.9990)	1.15E-05x-4.16 (r = 0.9970)	8.78E-06x-2.73 (r = 0.9990)	9.61E-06x-2.15 (r = 0.9991)	8.55E-06x-2.45 (r = 0.9992)
EP	1.06E-05x-1.77 (r = 0.9996)	8.73E-06x-1.63 (r = 0.9997)	1.19E-05x-3.54 (r = 0.9980)	9.09E-06x-2.27 (r = 0.9994)	1.10E-05x-3.34 (r = 0.9991)	8.77E-06x-1.74 (r = 0.9996)
PP	1.09E-05x-0.85 (r = 0.9999)	9.11E-06x-0.82 (r = 0.9999)	1.13E-05x-2.27 (r = 0.9990)	9.45E-06x-1.44 (r = 0.9996)	1.13E-05x-2.17 (r = 0.9995)	9.19E-06x-0.99 (r = 0.9998)
BP	1.14E-05x-0.48 (r = 0.9999)	9.60E-06x-0.58 (r = 0.9999)	1.17E-05x-1.89 (r = 0.9993)	9.93E-06x-1.16 (r = 0.9999)	1.17E-05x-1.59 (r = 0.9998)	1.01E-05x-2.93 (r = 0.9997)
BAol	6.90E-04x-35.7 (r = 0.9994)	6.13E-04x-10.72 (r = 0.9998)	**	5.95E-04x-6.88 (r = 0.9999)	5.77E-04x-3.40 (r = 0.9999)	5.93E-04x-8.63 (r = 0.9999)

* abbreviations see text. ** - not calibrated due to non-complete separation of benzyl alcohol with benzoic acid (R = 1.0).

Table 6. The linear relationship between analyte concentration (c) and peak area (x) for p-hydroxybenzoic acid, benzoic acid and benzaldehyde in elaborated chromatographic systems. The coefficients of correlation *r* given in brackets.

Substance*	Wavelength	$c = ax + b$					
		Column ODS-3			Column DP		
		pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
p-HBac	254 nm	9.13E-06x-0.24 (r = 0.9999)	8.82E-06x-0.11 (r = 0.9999)	8.58E-06x-0.17 (r = 0.9999)	7.83E-06x-0.08 (r = 0.9999)	8.97E-06x-0.26 (r = 0.9999)	7.71E-06x-0.09 (r = 0.9999)
Bac	220 nm	1.29E-05x-0.14 (r = 0.9999)	1.06E-05x-0.06 (r = 0.9999)	**	1.07E-05x-0.13 (r = 0.9999)	1.36E-05x-0.24 (r = 0.9999)	9.87E-06x-0.05 (r = 0.9999)
BAld	254 nm	9.59E-06x-0.24 (r = 0.9999)	1.07E-05x-0.06 (r = 0.9999)	**	9.31E-06x-0.29 (r = 0.9999)	1.06E-05x-0.40 (r = 0.9998)	8.71E-06x-0.11 (r = 0.9999)

* abbreviations see text. ** - not calibrated due to non-complete separation of benzyl alcohol with benzoic acid (R = 1.0).

Table 7. Times of retention of selected medicinal substances and their impurities in the elaborated chromatographic systems.

Substance*	Retention time [min]					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
C	2.7	6.4	40.2	6.2	26.3	7.0
pCB-ol	23.4	25.2	5.9	7.2	10.6	15.4
pCBP-on	49.8	53.17	7.2	9.0	17.7	27.3
Imp. A	2.0	4.9	50.6	9.6	30.1	21.2
D	28.1	18.4	na	5.3	11.1	37.7
Imp. A	23.9	23.8	na	8.0	10.1	14.5
Imp. B	66.1	63.2	na	10.8	19.1	30.0
Imp. C	30.1	29.5	na	8.4	11.7	17.2
Imp. E	3.9	4.3	na	4.1	3.8	4.5
T	2.7	71.1	na	15.8	60.1	62.9
Imp. A	1.7	24.0	na	5.9	11.7	8.2
Imp. B	2.6	68.2	na	15.5	64.0	61.0
Imp. E	1.9	>120	na	>80	>120	>120
E	1.7	2.3	15.2	4.3	6.3	4.9
Imp. A	1.7	2.3	14.5	4.5	7.3	5.3

* abbreviations see text. na – not analyzed (due to non-complete separation of benzyl alcohol with benzoic acid).

acid n-butyl ester exposed to light (254 nm) was also investigated (13).

The European Pharmacopoeia specifies the permissible amount of impurities in parabens and recommends the HPLC method for testing of parabens purity. However, there are no guidelines on their levels in medicinal products. They may not be considered as unknown impurities, as they often exceed the level of 0.2% adopted in line with ICH guidelines.

For determination of benzyl alcohol and testing of its purity the European Pharmacopoeia recommends a GC method. However, this method may not be sufficient, therefore a different method for testing of purity and determination of active substance should be applied. Simultaneous analysis with use of the two methods increases the costs.

The purpose of this paper was to elaborate HPLC methods for identification and quantitative determination of the parabens and benzyl alcohol in pharmaceuticals. It was assumed that the content of medicinal substances and preservatives as well as their purity, taking into consideration the impurities coming from the active substance as well as from the preservatives, would be determined in one chromatographic system.

For the testing the following were selected: expired preparations (Amertil, Effortil, Difadol) – their content of preservatives and their impurities had been determined earlier, during their period of validity – and a multi-dose preparation in its period of validity (Tenasil cream) – the amount of benzyl alcohol oxidation products may increase after opening of this preparation. This compilation allows estimation of the products of preservatives decomposition that are formed when storing under normal conditions as well as their rate of formation, and may facilitate determination of limits for these impurities.

EXPERIMENTAL

Test materials

LGC standards: methyl (**MP**), ethyl (**EP**), propyl (**PP**) and butyl (**BP**) p-hydroxybenzoates (parabens); p-hydroxybenzoic acid (**p-HBac**); benzyl alcohol (**BAol**); benzaldehyde (**BAld**); benzoic acid (**Bac**); certizine dihydrochloride (**C**), p-chlorobenzophenone (**p-CBPon**), p-chlorobenzhydrol (**p-CBHol**), A impurity acc. to Eu. Ph.; diclofenac sodium (**D**) and A, B, C and E impurities acc. to Eu. Ph.; etilefrine hydrochloride (**E**) and A

Table 8. Results of preservatives decomposition products determination (content expressed as percentage in relation to the parabens or benzyl alcohol content) in selected medicinal products with the use of elaborated chromatographic systems.

Assay [%]	Effortil				Amerтил			
	Column ODS-3 pH ~3	Column DP pH ~5	Column HS F5 pH ~3	Column HS F5 pH ~5	Column ODS-3 pH ~3	Column DP pH ~5	Column HS F5 pH ~3	Column HS F5 pH ~5
p-HBAc*	9.82	10.67	9.81	10.02	0.41	0.45	–**	0.43
Assay [%]	Difadol				Tenasil			
	Column ODS-3 pH ~3	Column DP pH ~5	Column HS F5 pH ~3	Column HS F5 pH ~5	Column ODS-3 pH ~3	Column DP pH ~5	Column HS F5 pH ~3	Column HS F5 pH ~5
BAc*	< LOQ	< LOQ	no	< LOQ	< LOQ	< LOQ	no	< LOQ
BAId*	0.03	0.03	0.03	0.03	0.26	0.26	no	0.25

* abbreviations see text. no – not determined. LOQ – limit of quantitation. **note: p-HBAc peak did not separate completely from the placebo peak.

impurity acc. to Eu. Ph.; terbinafine hydrochloride (T) and A, B and E impurities acc. to Eu. Ph.

Preparations containing: cetirizine dihydrochloride and methyl and ethyl p-hydroxybenzoates – Amerтил syrup 1 mg/mL s. CP8001, expiry date: 12. 2009 (Biofarm Sp. z o.o.); diclofenac sodium and benzyl alcohol – Difadol, solution for intramuscular injections 25 mg/g, s. 01AK1104, expiry date: 11. 2007 (WZF Polfa); etilefrine hydrochloride and methyl and propyl p-hydroxybenzoates – Effortil oral drops 7.5 mg/g, s. 433452D, expiry date: 09. 2009 (Boehringer Ingelheim); terbinafine hydrochloride and benzyl alcohol – Tenasil cream s. 010709, expiry date: 07. 2012 (Pharmaceutical Laboratory HOME OFARM sp. z o.o.).

The preparations were previously tested in November and December 2010.

HPLC reagents of high purity: acetonitrile (Rathburn, Scotland); deionized water (Millipore); 85% phosphoric acid; KH_2PO_4 and K_2HPO_4 (AppliChem).

HPLC columns: used for reconstruction of pharmacopoeia monograph for parabens: Zorbax Rx C18, 5 mm, 150×4.6 mm, Agilent; used for elaboration of new methods: Supelcosil LC-DP, 5 mm, 250×4.6 mm, Supelco; Inertsil ODS-3, 5 mm, 250×4.6 mm, MZ-Analysentechnik GmbH; Discovery HS F5, 5 mm, 250×4.6 mm, Supelco.

Apparatus: PC controlled liquid chromatograph with SPD-10AVvp UV detector, LC-10ATvp pumps and DGU-14A mobile phase degasser, SCL-10AVvp controller, automatic sample injector SIL-10AVvp – manufactured by Shimadzu, Japan.

Solutions used for HPLC: solvent for solutions preparation: acetonitrile and water mixture 45 : 55 (v/v); standard solutions for determination of calibration curves: paraben solutions in a range of concentrations from 10 to 200 $\mu\text{g/mL}$; solutions of p-hydroxybenzoic acid in a range of concentrations 0.03–50 $\mu\text{g/mL}$; solutions of benzyl alcohol in a range of concentrations 10–1700 $\mu\text{g/mL}$; solutions of benzaldehyde in a range of concentrations 0.05–75 $\mu\text{g/mL}$; solutions of benzoic acid in a range of concentrations 0.03–50 $\mu\text{g/mL}$; placebo solutions: weigh about 50 mg of every excipient and dissolved in solvent; tested solutions obtained from pharmaceutical preparations: Amerтил – concentration of medicinal substance ca. 0.1 mg/mL, concentration of MP ca. 0.135 mg/mL, concentration of PP ca. 0.015 mg/mL; Difadol – concentration of medicinal substance ca 1 mg/mL, concentration of BAol ca. 1.4 mg/mL; Effortil – concentration of medicinal substance ca. 0.75 mg/mL, concentration of MP ca. 0.07 mg/mL, concentration of PP ca. 0.03 mg/mL;

Table 9. Comparison of retention times of active substance, preservatives and products of their decomposition in selected preparations obtained by elaborated chromatographic systems.

Substance*	Effortil					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
E	1.7	2.3	15.2	4.3	6.3	4.9
Imp. A	1.7	2.3	14.5	4.5	7.3	5.3
p-HBAc	3.6	3.5	3.8	3.5	4.0	3.9
MP*	5.9	5.9	4.6	4.5	6.0	5.9
PP*	11.5	11.3	5.8	5.4	9.3	9.0
Substance*	Amertil					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
C*	2.7	6.4	40.2	6.2	26.3	7.0
Imp. A	2.0	4.9	50.6	9.6	30.1	21.2
p-CBoI	23.4	25.2	5.9	7.2	10.6	15.4
p-CBPon	49.8	53.17	7.2	9.0	17.7	27.3
p-HBAc	3.6	3.5	3.8	3.5	4.0	3.9
MP	5.9	5.9	4.6	4.5	6.0	5.9
PP	11.5	11.3	5.8	5.4	9.3	9.0
Substance*	Didafol					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
D	28.1	18.4	na	5.3	11.1	37.7
Imp. A	23.9	23.8	na	8.0	10.1	14.5
Imp. B	66.1	63.2	na	10.8	19.1	30.0
Imp. C	30.1	29.5	na	8.4	11.7	17.2
Imp. E	3.9	4.3	na	4.1	3.8	4.5
BAolc	5.1	5.1	4.4	4.3	5.	5.1
BAc	5.6	4.2	4.5	3.7	5.6	6.8
BAld	8.4	7.9	5.2	5.0	7.8	7.7
Substance*	Tenasil					
	Column ODS-3		Column DP		Column HS F5	
	pH ~3	pH ~5	pH ~3	pH ~5	pH ~3	pH ~5
T	2.7	71.1	na	15.8	60.1	62.9
Imp. A	1.7	24.0	na	5.9	11.7	8.2
Imp. B	2.6	68.2	na	15.5	64.0	61.0
Imp. E	1.9	> 120	na	> 80	> 120	> 120
BAolc	5.1	5.1	4.4	4.3	5.	5.1
BAc	5.6	4.2	4.5	3.7	5.6	6.8
BAld	8.4	7.9	5.2	5.0	7.8	7.7

* abbreviations see text. na – not analyzed (due to non-complete separation of benzyl alcohol with benzoic acid). Imp. A, B, C, E – impurity for each substance by Ph. Eur.

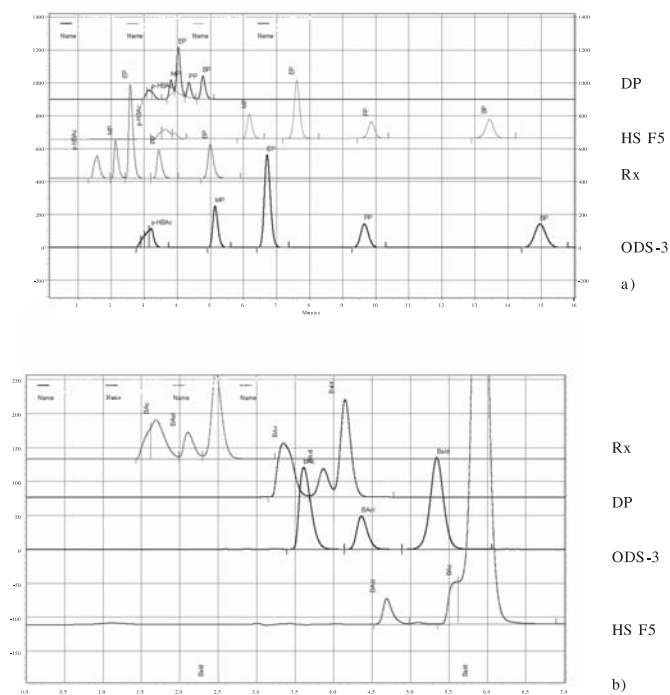


Figure 1. Chromatograms in chromatographic system acc. to Eur. Ph. monographs for parabens on the columns: Zorbax Rx C18 (recommended by Eur. Ph.) and ODS -3, HS F5, DP
 a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-BAc)
 b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

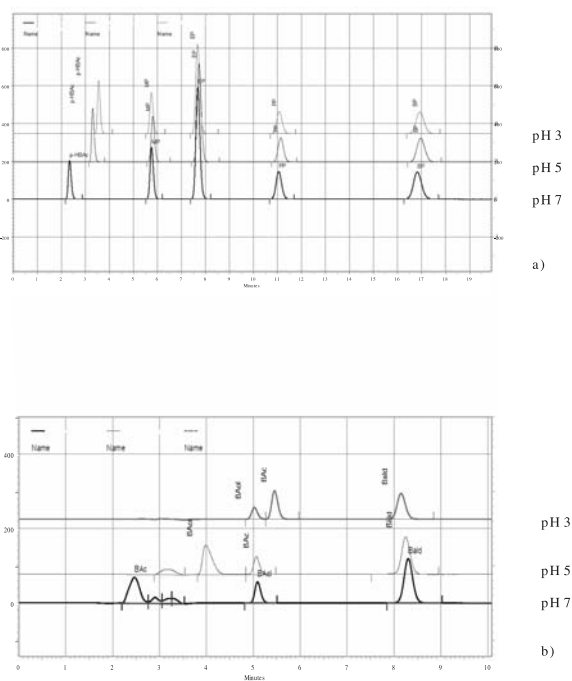


Figure 2. Chromatograms in elaborated chromatographic systems on the column ODS-3
 a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
 b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

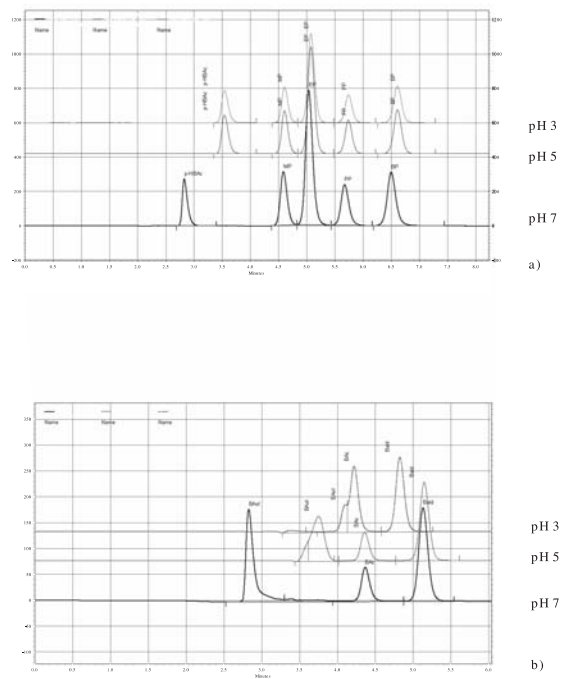


Figure 3. Chromatograms in elaborated chromatographic systems on the column DP
 a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
 b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

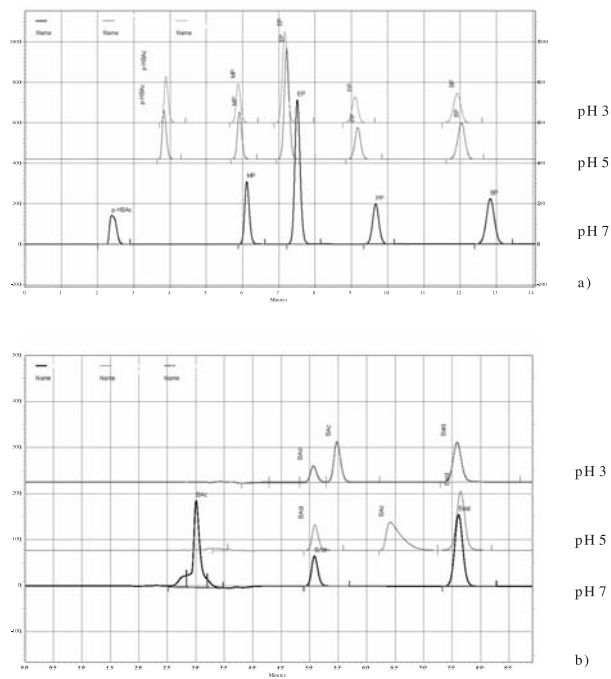


Figure 4. Chromatograms in elaborated chromatographic systems on the column HS F5
 a) parabens (MP, EP, PP, BP) and p-hydroxybenzoic acid (p-HBAc)
 b) benzyl alcohol (BAol), benzaldehyde (Bald) and benzoic acid (BAc)

Tenasil – concentration of medicinal substance ca. 1 mg/mL, concentration of BAol ca. 1 mg/mL; solutions for identity confirmation of: certizine dihydrochloride and impurities; diclofenac sodium and impurities, etilefrine hydrochloride and impurities, terbinafine hydrochloride and impurities.

RESULTS AND DISCUSSION

A solution was prepared of standard substances: methyl, ethyl, propyl and butyl parabens and p-hydroxybenzoic acid in the acetonitrile : water mixture (45 : 55, v/v) with the concentrations of approx. 100 µg/mL (parabens) and 25 µg/mL (acid). This solution was used to reproduce the HPLC system acc. to Eu. Ph. for testing of the purity of parabens. Then, the solution of the following standard substances was prepared: benzyl alcohol, benzaldehyde and benzoic acid in the mixture of acetonitrile : water (45 : 55, v/v) in the concentrations of 800, 40 and 25 µg/mL, respectively, and it was verified whether the abovementioned HPLC system is suitable for determination of benzyl alcohol and its decomposition products (Tab. 1). The retention times for all standard substances are within the range from 1.7 to 5.7 min. Due to the presence of excipients in preparations, such retention times are being too short. Therefore, the system was modified by application of columns with a different filling material (DP, ODS-3, HS F5 columns) than the one recommended by Eur. Ph. (Zorbax Rx C18 column) with the same mobile phase, i.e., the mixture of 0.05 M KH_2PO_4 and methanol in the proportion of 35 : 65 (v/v). It turned out that the peak of p-hydroxybenzoic acid was divided and was not suitable for quantitative determinations. In the case of benzyl alcohol and the products of its decomposition on the ODS-3 and HS F5 columns the obtained separation was suitable, but the peaks were relatively wide and asymmetric (Tab. 1, Fig. 1).

For elaboration of new methods the columns with variable filling materials were selected: ODS-3 – alkyl groups bonded to spherical silica, with large specific surface (450 m²/g); HS F5 – pentafluorophenyl propyl groups bonded to spherical silica, combining the features of reversed and normal phases, allowing for delaying of polar compounds retention with no delay in non-polar substances retention; DP – diphenyl groups bonded to spherical silica, ensuring higher selectivity for aromatic groups, with moderate polarity.

Next, three other mobile phases of various pH were prepared: pH~3 – a mixture of acetonitrile with water in proportions of 45 : 55 (v/v) with addition of

85% phosphoric acid in the amount of 0.2 mL per one litre of the phase; pH~5 – a mixture of acetonitrile with 0.025 M solution of KH_2PO_4 in a proportion of 45 : 55 (v/v); pH~7 – a mixture of acetonitrile with a solution containing 0.0125 M of KH_2PO_4 and 0.0125 M of K_2HPO_4 in a proportion of 45 : 55 (v/v).

The tests with the use of the mobile phases were carried out on each of the columns. The remaining conditions of analysis have always been the same: temperature of column operation: 40°C; flow rate of the mobile phase: 1 mL/min; UV detection – 220 nm and 254 nm; sample dose volume – 20 µL.

The results for parabens and benzyl alcohol and their degradation products recorded for all columns and mobile phases are given (Tabs. 2–4 and Figs. 2–4). The mobile phase of pH ~7 was eliminated on all the columns because p-HBac and BAc acids were eluted too fast and their peaks are often divided and may overlap the solvent peaks or placebo peaks in case of preparations analysis. Moreover, no determinations of benzyl alcohol and its decomposition products on the DP column, pH ~3 phase could be made because of non-complete separation of BAol and BAc peaks (Tab. 3, Fig. 3).

For the rest of combinations column–mobile phase, the calibration curves, relationship between the analyte concentration expressed in µg/mL and the peak area, analyses were performed. The concentration ranges were selected as those that are usually applied for determination of the compounds in pharmaceutical preparation: parabens (10–200 µg/mL), benzyl alcohol (10–1700 µg/mL), p-hydroxybenzoic acid (0.03–50 µg/mL) and benzaldehyde (0.05–75 µg/mL) and benzoic acid (0.03–50 µg/mL) (Tabs. 5, 6). Limits of detection were determined at the level of 0.01 µg/mL by determining the S/N ratio. Next, the samples, which were prepared by dissolving the medicinal products in the mixture acetonitrile and water (45 : 55, v/v), have been examined in the selected systems. Retention times of medicinal substances and their impurities are given in Table 7.

Selection of columns with other filling material (DP, ODS-3, HS F5) and adequate mobile phases ensured proper selectivity (all values $R_s > 2$), shape of the peaks (Tabs. 2–4, Figs. 2–4) as well as sensitivity (0.01 µg/mL). It was proven that pH of the mobile phase influences the time of retention of p-hydroxybenzoic and benzoic acids (Tabs. 2–4, Figs. 2–4) and in the case of products testing – the time of retention of medicinal substances (Tab. 7). The systems that are most suitable for quantitative analysis of decomposition products were selected.

The selected systems were used to determine decomposition products of preservatives in medicinal products with no impurities indicated in their specifications. The results obtained in different systems were generally comparable (Tab. 8). For Amertil preparation, tested in the phase with a pH ~3, the results obtained only with the use of ODS-3 column have been presented, because in the case of DP and HS-F5 columns the p-HBac peak is not entirely separated from the placebo peaks. Tenasil and Difadol preparations that contain benzyl alcohol were not tested on DP column with the pH ~3 phase due to the non-complete separation of BAol and BAc peaks.

Table 9 includes the comparison of retention times of active substance, preservative and their decomposition products.

CONCLUSIONS

The abovementioned results show that it is not possible to simply transfer the pharmacopoeial method of parabens purity testing to the columns with different filling material (Tab. 1, Fig. 1) and to apply these methods in the testing of purity of preparations containing parabens or benzyl alcohol. This method is not applicable in medicinal products due to too short retention times and a possibility that the peaks from placebo interfere with the peaks of p-hydroxybenzoic acid.

Therefore, new HPLC systems characterized with appropriate selectivity (all the R_s values > 2) and sensitivity (LOD approx. 0.01 µg/mL) have been elaborated. They allow for testing of parabens purity in the presence of parabens hydrolysis product (p-hydroxybenzoic acid) and testing of benzyl alcohol purity in the presence of its oxidation products (benzaldehyde and benzoic acid). Assessment of purity of the preservatives may be performed with satisfactory precision. These systems may also be used for quantitative determination of preservatives, as it was demonstrated that there is a linear dependence between the analyte content and the peak area in terms of concentrations usually used for determination of preservatives content in medicinal products.

The tests performed on the expired preparations revealed that the amount of preservatives

decomposition products exceeded 0.2% (limit value for unknown impurities according to ICH), and confirmed the need of their determination.

The use of columns with various filling materials and mobile phases with a pH ranging from 3 to 7 makes it possible to match the system to the composition of an investigated product.

The proposed HPLC systems shall be optimized for testing other medicinal products, containing the preservatives.

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IDENTIFICATION OF SIMILARITIES IN METALLIC CONTENT OF HERBAL INFUSIONS USING NON-LINEAR APPROACH

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Abstract: The content of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn was determined, using flame atomic absorption spectrometry in infusions made from commercial herbal mixtures readily available in herbal stores. ANOVA test revealed that the metal contents did not differ considerably in most of infusions. The scatter plot of principal component analysis (PCA) designated infusions characterized by similar contents of trace elements. It was possible to indicate distinctive metal concentrations in Pyrosan, Septosan and Urosan infusions as they vastly contrasted with others. The levels of Mn, Zn, Cd were characteristic for Pyrosan infusions, while Cr, Fe, Cd were specific to Septosan, and Mn, Zn, Fe to Urosan. Self-organizing maps did not fully correspond with PCA results because PCA classified samples relying mainly on apparent similarities in metal contents.

Keywords: herbal infusions, principal component analysis, self-organizing maps, trace metals

Herbal remedies have been used for centuries in order to sustain good health, cure common illnesses or treat various health conditions. Nowadays, even though conventional medicine is developing at a high rate with a growing number of drugs obtained by chemical synthesis, the interest in herbalism and phytopharmaceuticals has not diminished (1). The increased use of herbal medicines is based on the belief that they are natural and therefore safe, so fewer side-effects will be produced. In addition, most of them are freely available (2).

One of the most popular forms of herbal drugs to be taken by patients are the mixtures composed of several medicinal plant raw materials coming from various species of plants. They are usually available in small sachets and administered by making a tea to be drunk twice or three times a day in order to cure various ailments such as: infections, diabetes, rheumatism, inflammatory diseases, influenza etc. (3). However, apart from active organic ingredients responsible for therapeutic activities (4), medicinal herbs also contain trace elements. Some of them have potential synergistic or antagonistic properties to pharmacological effects of organic compounds, but others are simply toxic for human health (5).

In recent years, many papers have been published on analysis of trace elements in various herbal products, mostly for quality control reasons (6–9)

according to WHO recommendations (10). However, in many cases, along with determination of metals levels in herbals, different chemometric techniques were involved (11–17). The reason for that was the fact that samples were described by a large number of metal concentrations, so it was difficult to identify any intercorrelations between those metals straightforward. The most commonly used method was principal component analysis (PCA) (12, 14), most often accompanied by cluster analysis (CA) (11, 13, 16) and at times, by linear discriminant analysis (LDA) (15, 17). These chemometric techniques have been applied to investigation of the contents of heavy metals in herbs or herbal teas in order to classify and examine any relationships between these metals (15), identify similarities regarding mineral and trace metal contents of spices and herbs (17), quantify the content of various elements possibly responsible for some therapeutic properties of *Echinacea purpurea* (11), recognize potential anthropogenic contamination sources of herbal drugs (12, 13), establish standardization and quality control procedures for crude drugs (14), and differentiate herbal raw materials belonging to different plant families according to metal contents (16).

After reviewing the above literature, it was concluded that mostly applied chemometric meth-

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ods used for identification of similarities or correlations between metallic content in herbals were based on linear modeling. Having assumed that interrelationships between chemical constituents occurring in nature are rarely of linear character, it was decided to explore relationships in elemental content of herbal infusions using non-linear modeling. In this paper, nonlinear approach was proposed using self-organizing maps (SOMs) also known as Kohonen neural networks.

SOMs are intended for unsupervised learning tasks similarly to PCA and CA. Contrary to supervised learning, the training data set contains only inputs and SOM attempts to learn the structure of the data on the basis of these variables. SOM is capable of recognizing clusters of data and relating classes similar to each other, which makes understanding of the data feasible. Once classes of data are identified, they are tagged and the network becomes ready for classification tasks. Kohonen networks can be also applied for classifications where the output classes are known in advance. In such circumstances, they are able to highlight similarities between groups (18, 19).

In this study, output classes (herbal mixtures) and individual samples were known, therefore, the subject was to discover the characteristic differences between the classes and similarities within the classes with regard to the concentrations of some trace elements. Therefore, the heavy metals content (Ni, Cr, Cu, Fe, Mn, Zn, Pb and Cd) was determined using flame atomic absorption spectrometry in infusions prepared from commonly available herbal mixtures. Furthermore, ANOVA, PCA and SOMs were employed to verify if there are any similarities regarding the concentration of the metals. It was decided to establish if the presence of certain herbal constituent in a mixture can be assigned to the levels of some trace elements. The results of the mapping of the infusions performed by SOMs were compared to widely applied PCA.

EXPERIMENTAL

Herbal material

Herbal mixtures produced by "Herbapol" commercially available in drug or herbal stores were involved in the study. The total number of infusions

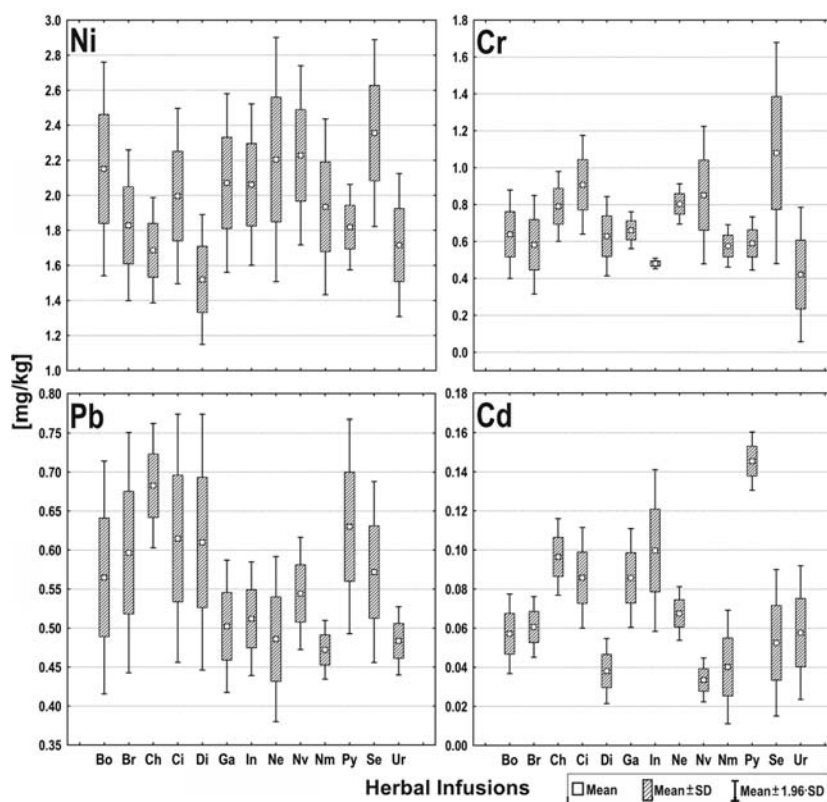


Figure 1. The box-whiskers plot of Ni, Cr, Pb, Cd content in herbal infusions

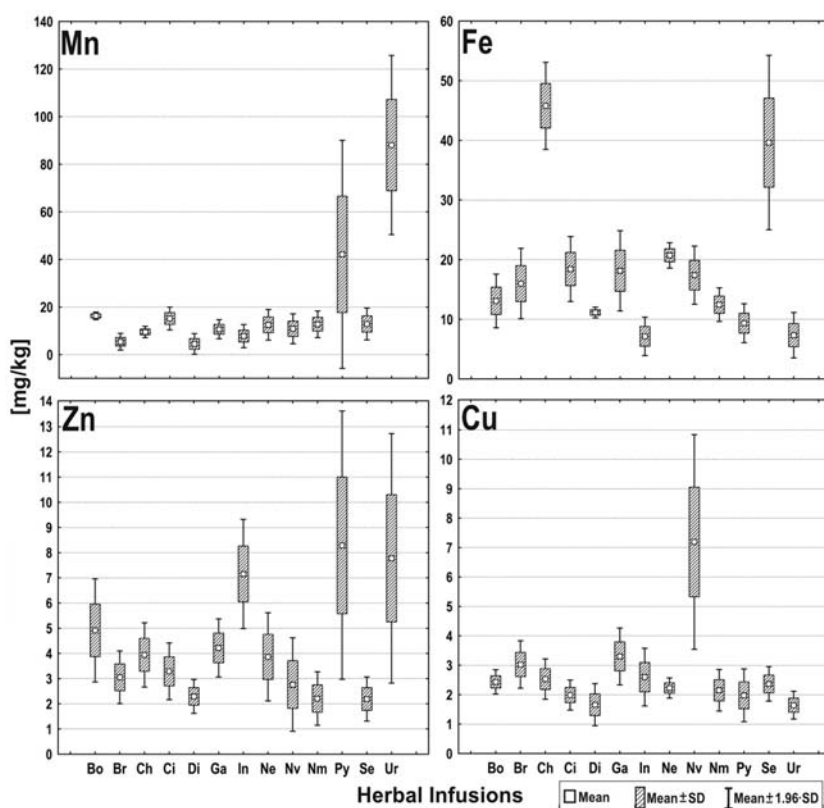


Figure 2. The box-whiskers plot of Mn, Fe, Zn, Cu content in herbal infusions

was 73, which were prepared from 13 different mixtures. The analyzed herbal preparations are listed below in alphabetical order with two letter abbreviations and samples numbers provided in parentheses: Bobofen (Bo, no. 1–4), Bronchial (Br, no. 5–12), Cholagoga II (Ch, no. 13–16), Circulosan (Ci, no. 17–20), Diabetosan (Di, no. 21–24), Gastrosan (Ga, no. 25–32), Infektoten (In, no. 33–35), Nervinum (Ne, no. 36–38), Nervosan (Nv, no. 39–44), Normosan (Nm, no. 45–53), Pyrosan (Py, no. 54–56), Septosan (Se, no. 57–64) and Urosan (Ur, no. 65–73).

The herbal mixtures consist of various herbal raw materials. Some of them are present in different mixtures e.g., melissa leaf in 5 mixtures, peppermint leaf and chamomile in 4 and hop in 3. Several mixtures include one common raw material e.g., birch leaf – Urosan and Pyrosan, yarrow herb – Cholagoga II and Nervosan, lime inflorescence – Bronchial and Pyrosan etc. The mixtures are mostly used as anti-inflammatory, analgesic, diaphoretic, sedative, antipyretic, diuretic, spasmodic, diastolic and cholagogic.

Sample preparation

All batches of herbal mixtures were homogenized in a water-cooled grinder Knifetec 1095 (Foss Tecator, Höganäs, Sweden) at 20°C for 30 s. After that, an accurately weighed homogenized sample in the amount of 10 g was put into a beaker and poured onto with 200 mL of boiling deionized water. The beaker was covered with a watch glass and left for 15 min to steep. After that time, infusion was cooled, strained using MN 640d ashless filter paper (Macherey-Nagel, Duren, Germany) into a 200 mL volumetric flask and filled up with deionized water. Each infusion was prepared three times.

Trace elements determination

The content of Cd, Cr, Cu, Fe, Pb, Mn, Ni and Zn was determined using spectrometer SpectraAA 250 Plus (Varian, Australia) in flame atomic absorption mode. The accuracy and precision of the procedure were established on the basis of several measurements of the Certified Reference Material CTA-VTL-2 (Virginia Tobacco Leaves), manufactured by The Institute of Nuclear Chemistry and Technology

(Warszawa, Poland). The recovery levels were in the range of 89.1–96.0% depending on the element. The lowest recovery was obtained for Cr and the highest for Zn.

Software

All calculations were made by means of a statistical software – STATISTICA 9.0, (Statsoft Inc., USA).

RESULTS AND DISCUSSION

Results of analysis of variance

Categorized box plots showing the mean contents (small squares) of particular elements in the herbal infusions are presented in Figures 1 and 2. The mean value is surrounded by rectangle, which denotes ± 1 times the standard deviation (SD), while whiskers represent a 95% confidence interval defined as the mean ± 1.96 times the SD, in view of the fact that the distribution is normal.

As it is shown in the plots, most of infusion samples demonstrate similar levels of 8 analyzed

elements, in spite of the fact that commercial herbal mixtures contain different medicinal plant raw materials in varied proportions. However, some water extracts prepared from Pyrosan, Septosan and Urosan mixtures were presented as exceptions. The observations were statistically confirmed by ANOVA test ($p < 0.05$), which pointed to significant differences between mean contents of certain metals. In order to determine which group means differ from one another, Tukey's multiple comparison test, for unequal number of cases, was used. The results indicated that the fewest statistically significant differences between mean contents of elements in infusions were observed mainly in the case of Ni and Cr. The levels of Pb, Cu, Mn and Zn did not present a considerable number of meaningful dissimilarities as well. The elements which differentiated between infusions to the largest extent were Cd and Fe.

The analysis of Ni and Cr in infusions showed that only Septosan infusions statistically differed from other extracts (mean content 2.35 mg Ni/kg and 1.08 mg Cr/kg). The levels of Pb distinguished extracts of Normosan and Chologoga II with the

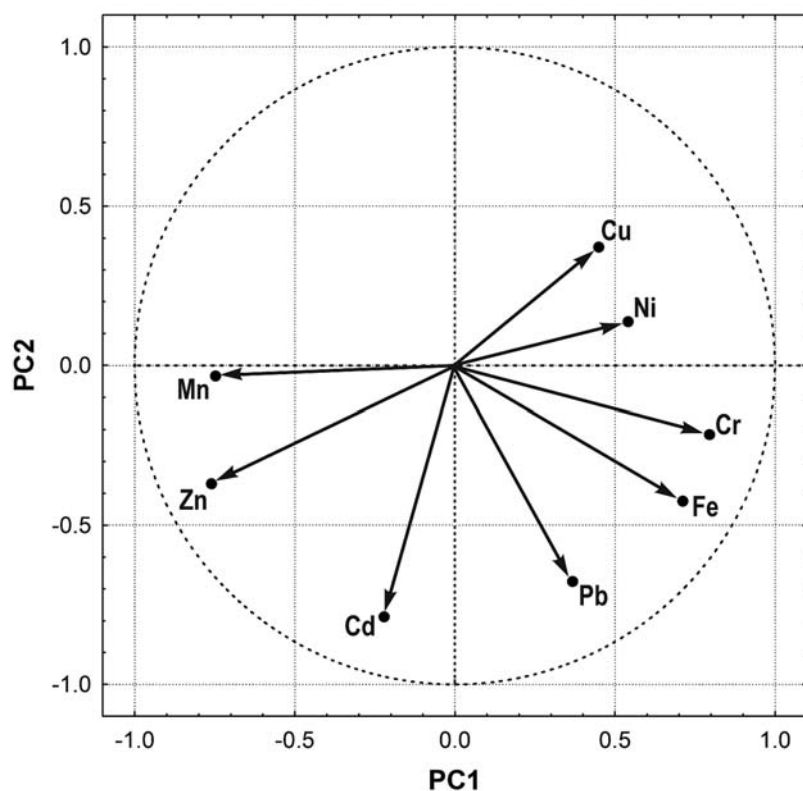


Figure 3. The plot of component loadings of trace elements

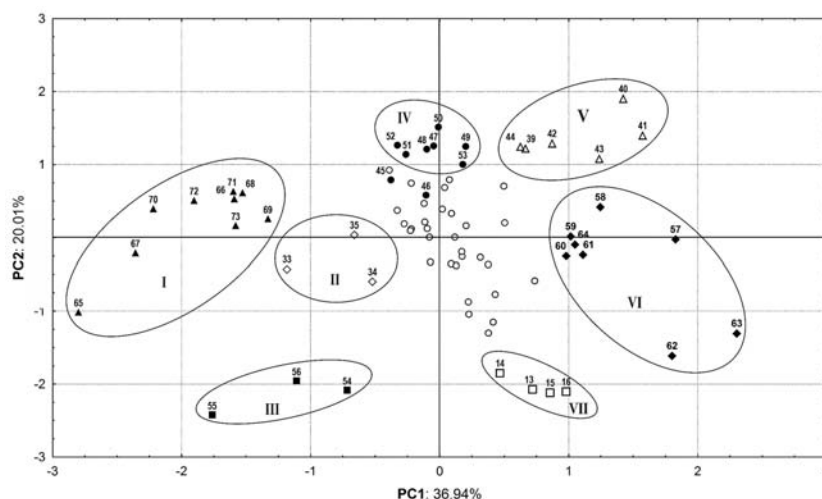


Figure 4. The scatter plot of PCA scores (PC1 vs. PC2) for herbal infusions

concentration of Pb amounted to 0.472 and 0.682 mg/kg, respectively. With regard to Cd, it was noted that despite numerous statistically significant differences between concentrations of both metals in various infusions, the most noticeable one was observed in water extracts prepared from Pyrosan (0.145 mg Cd/kg). Gastrosan, Cholagoga II and Infektoten infusions showed no differences between each other considering Cd content, while being dissimilar to other extracts. The mean contents of Cd were 0.086, 0.096 and 0.099 mg/kg, respectively.

The mean levels of Mn were found to be characteristic for Pyrosan (ca. 42 mg/kg) and Urosan infusions (88 mg/kg), while Fe for Septosan and Cholagoga II, containing on average 39.61 and 45.78 mg/kg. The amount of Zn clearly distinguished Infektoten, Urosan and Pyrosan. The mean concentrations of Zn were varied between 7.17 and 8.29 mg/kg. Cu pointed to the extracts of Nervosan (ca. 7 mg/kg).

Results of principal component analysis

The results of PCA showed that PC1 explained 36.94% of overall variance, PC2 – 20.01% and PC3 – 14.12%. Taking into consideration first two PCs, the amount of lost information was equal to 43.05%. The degree of influence on the interpretation of two-dimensional distribution of infusions is determined by loadings values (Fig. 3), which characterize relations between the levels of metals and principal components. The loadings indicate that extracts with higher concentration of Cu and Ni are to be located in upper right side of the PCA scatter plot, as shown

in Figure 4. The extracts described by higher levels of Cr, Fe and Pb were shifted towards its lower right side, while the amount of Mn, Zn and Cd was responsible for directing infusions to the lower left side of the plot. In the distribution of analyzed infusions in PCA plot (Fig. 4), seven separated groups were distinguished, denoted by Roman numerals from I to VII. These groups contain infusions prepared from mixtures of the same composition. The closer the samples are located to each other, the more similar are the levels of trace metals determined in them.

The most numerous group of extracts, made from Urosan mixture, was situated on the left side of the plot (group I). The distinctive feature of these samples was a high concentration of Mn, above 64 mg/kg. The extracts (no. 65) and (no. 67) were separated from other samples as a result of the greatest content of Zn, 13.24 and 9.89 mg/kg, respectively. Close to the center of the plot, in group II, there are three infusions of Infektoten (no. 33–35). No strong dominance of any element was noticed. The samples had similar levels of Cr (0.470–0.497 mg/kg), Zn (above 5.95 mg/kg) and Cd (above 0.076 mg/kg). Group III is composed of Pyrosan extracts (no. 54–56), with the highest concentration of Cd, exceeding 0.138 mg/kg, and relatively high level of Mn, above 19.44 mg/kg. Although Mn content is the largest when related to other extracts (excluding Urosan samples), the differences between individual samples are too great (ca. 1.5–3.5-fold). Therefore, it is difficult to regard the concentration of Mn as a characteristic attribute of Pyrosan infusions.

The infusions found in groups I–III are in many cases characterized by similar concentrations of trace elements. It comes to attention that mutual medicinal plants raw materials are included in their composition. Infektoten and Pyrosan contain black elder flower and willow bark, while the common ingredient for Pyrosan and Urosan is birch leaf.

Group IV represents Normosan infusions (no. 45–53). The position of the samples was caused by comparable amounts of Fe (10.56–14.45 mg/kg), Cr (0.474–0.667 mg/kg) and Pb below 0.49 mg/kg. Unfortunately, three samples representing other infusions are characterized by similar concentrations of those metals. In near vicinity group V was positioned. This group includes Nervosan infusions (no. 39–44), which are characterized by the highest level of Cu, in the range of 4.01–9.35 mg/kg, and low content of Cd, less than 0.042 mg/kg.

The extracts of Septosan created group VI. Their most distinctive attribute is high concentration

of Fe (28.48–50.09 mg/kg). In two samples of Septosan (no. 62 and 63), the highest levels of Cr – 1.240 and 1.721 mg/kg, pushed them to the lower side of the PCA plot. Cholagoga II infusions, characterized by the highest concentration of Fe (41.44 to 50.49 mg/kg), formed group VII located in lower right side of PCA plot. Both Septosan and Cholagoga II mixtures contain peppermint leaf. This plant raw material is present in Gastrosan and Normosan as well. However, in PCA they were placed in the middle of the plot together with infusions prepared from other mixtures. Cholagoga II mixture also comprises yarrow herb similarly to Nervosan samples, which are on the same side of PCA plot.

Results of self-organizing maps

In further step of the research, the results of PCA were juxtaposed with those obtained by application of self-organizing maps. The mapping of

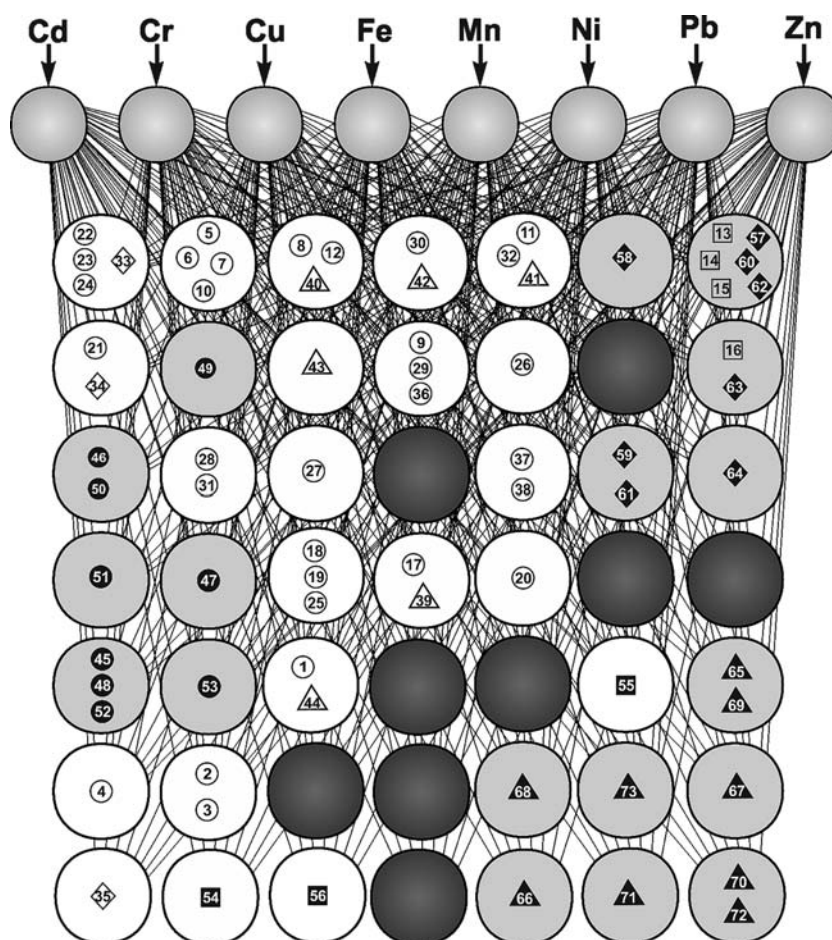


Figure 5. The distribution of herbal infusions on Kohonen map. (Neurons with no samples are marked dark grey).

infusion samples shown in Figure 5 did not distinguish as many consistent groups as PCA. It turned out that Nervosan extracts placed together in group V in PCA plot, were scattered all over Kohonen map, and its samples can be found in different distant neurons. It proves that despite the same composition of herbal mixture, there are substantial dissimilarities in the concentration of some elements. Furthermore, the level of Cu, quite different in comparison to other mixtures, was not recognized as a special attribute of all Nervosan extracts. A similar case was observed with regard to Pyrosan and Infektoten infusions, which formed two separate groups II and III in PCA score plot.

Although ANOVA test indicated differences in Cd levels in the extracts of both mixtures, the concentration of remaining elements was comparable. Two groups of infusions prepared from Chologoga and Septosan, containing the highest amount of Fe, as opposite to PCA, remained unseparated on the map. They were put together in neurons located in upper right corner of SOM (grey neurons). The difference in mean concentrations of Cd and Pb caused separation of these two groups of mixtures in PCA. Kohonen map, however, while taking into consideration the levels of all analyzed metals, did not identify significant distinctions, and placed them together.

Normosan infusions presented themselves as an interesting case. The analysis of variance did not reveal differences between Normosan and extracts of other mixtures. In spite of this fact, PCA distinguished these samples as group IV, though situated closely to other extracts. In contrast, infusions of Normosan were gathered in adjacent neurons on the left side of the Kohonen map, which clearly suggests a considerable resemblance within the group in regard to metals levels.

The infusions of Urosan, similarly to PCA, were also differentiated from others by SOM. They can be found in lower right corner (grey neurons). Undoubtedly, trace elements whose concentrations greatly affected such allocation of these extracts were Mn, Zn and, to a lesser extent, Fe.

CONCLUSIONS

The research showed that metals composition of water extracts, prepared from commercial herbal mixtures, is vastly similar. ANOVA test indicated that only in a small number of infusions, the content of elements considerably differed from each other. PCA indicated that the metals content can be a characteristic feature for some infusions. It was demon-

strated by the formation of several consistent groups. The concentrations of Mn, Zn, Cd in Pyrosan, Cr, Fe and Cd in Septosan and Mn, Zn, Fe in Urosan extracts, particularly varied from extracts prepared from the rest of herbal mixtures. Infusions, in which the mean content of only one metal determined their distinction from others, were also identified. It was Cu contents in Nervosan, and the levels of Fe in Chologoga II samples.

Non-linear modeling performed by Kohonen map managed to reveal more subtle similarities in metal contents in Urosan and Normosan, and to some extent – in Septosan and Chologoga II extracts. In this way, the results of ANOVA test were confirmed, pointing out to some simplifications made by PCA method, which considered only evident similarities in metal contents.

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DRUG BIOCHEMISTRY

EFFECTS OF VANADIUM COMPLEXES SUPPLEMENTATION ON
V, Cu, Mn, K, Fe, Zn, AND Ca CONCENTRATION IN STZ DIABETIC
RATS PANCREASMirośław Krośniak¹, Joanna Kowalska^{2,6}, Renata Francik^{3,4}, Ryszard Gryboś⁵ and Wojciech M. Kwiatek⁶¹Jagiellonian University Medical College, Department of Food Chemistry and Nutrition,
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Abstract: The objective of the study was to assess the effects of $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(2,2'\text{-bpy})] \times 8 \text{H}_2\text{O}$ (complex 1), $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(1,10'\text{-phen})] \times 5 \text{H}_2\text{O}$ (complex 2), $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(4,4'\text{-Me-2,2'}\text{-bpy})] \times 8 \text{H}_2\text{O}$ (complex 3), $[\text{V}^{\text{VO}}(\text{SO}_4)(1,10'\text{-phen})] \times 2 \text{H}_2\text{O}$ (complex 4), $[\text{V}^{\text{VO}}(\text{SO}_4)(2,2'\text{-bpy})] \times \text{H}_2\text{O}$ (complex 5), where: 2,2'-bpy = 2,2'-bipyridine, 1,10'-phen = 1,10'-phenanthroline, 4,4'-Me-2,2'-bpy = 4,4'-dimethyl-2,2'-bipyridine and a small insulin injection on V, Cu, Mn, K, Fe, Zn, and Ca concentration in the STZ (streptozotocin) diabetic rats pancreas during a 5-week treatment with the tested complexes. In all groups of animals metal concentration in the pancreas was investigated by means of Proton Induced X-ray Emission (PIXE) method. Maximum concentration of vanadium was observed in the pancreas for complex 5 ($1.69 \pm 0.09 \text{ mg/kg}$ dry weight), lower for complex 3 ($1.51 \pm 0.10 \text{ mg/kg}$ dry weight), and the lowest for complex 1 ($1.21 \pm 0.27 \text{ mg/kg}$ dry weight) supplementation. The influence of vanadium administration on other metals' concentration in the rats' pancreas was also investigated. All vanadium-tested complexes showed an increase of zinc concentration in the examined pancreas in comparison to the diabetic animals not treated with vanadium. The results were the highest for complex 1 and the lowest for complex 5. The concentration of Fe, Cu, Mn, K and Ca in the pancreas is not evidently influenced by administration of the vanadium complexes.

Keywords: pancreas, rats, PIXE, diabetes, vanadium, copper, manganese, potassium, iron, zinc, calcium

Diabetes, especially type 2, affects increasing numbers of people and creates a global threat to health and life (1). According to the World Health Organization, after the year 2020, the number of patients suffering from diabetes will be doubled. In this disease the irregular distribution of carbonate, resulting from the insulin deficiency in case of Type 1 diabetes and from insulin resistance in case of Type 2 diabetes is a characteristic event. The cause for this injury related to hyperglycemia is the formation of glycosylated proteins, glucose oxidation, and increased level of free fatty acids (2). This results in oxidative stress in the cells, as well as activation of oxidative and inflammatory signalling pathways,

which continue to damage the insulin-diarrhoea producing cells, what leads eventually to various complications of diabetes.

At present, the basic aim of the research is to identify and understand the exact mechanisms of the development of diabetes. The pancreas is a large gland located behind the stomach and next to the gallbladder. It produces digestive enzymes and hormones, including insulin and glucagon that help to regulate blood's sugar levels. It was also shown that the levels of indicators of oxidative stress firstly increase and then decrease after antioxidants supplementation (2). This suggests that chronic pancreatitis (CP) must involve a state of heightened free rad-

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ical mediated injury, and that damage apparently can be reversed in many people with antioxidant supplements. CP is a progressive inflammatory disease, that can lead to a loss of the function of the pancreas. As a result, people with CP can lose too much weight, suffer frequent diarrhoea, and development of diabetes or vitamin deficiencies. Patients with exocrine pancreatic insufficiency are at a greater risk of developing trace-element deficiencies as a result of malabsorption than patients with exocrine pancreatic sufficiency (1–3).

Dietary supplementation with micronutrients may be a complement to classical therapies for preventing and treating diabetic complications. Supplementation is expected to be more effective when a deficiency in these micronutrients exists. Some vitamins, such as vitamins E, C, and A and the carotenoids as well as trace elements such as selenium and zinc, have antioxidant properties (4). In diabetes, cells and tissues are damaged due to the imbalance between the production and the removal of free radicals. The effective biologic antioxidants for oxidative stress, such as α -lipoic acid, vitamin E and selenium, are effective in diminishing oxidative damage like membrane lipid peroxidation (5). However, zinc is involved in a multitude of these processes within the pancreas, including glucagon secretion, digestive enzyme activity, and insulin packaging, secretion and signalling. As a result of this extensive physiological contribution, deregulations of Zn metabolism within the pancreas impairs a multitude of key processes, including glycemic control (6, 7).

The prevalence of lower plasma concentrations of trace elements in patients with CP has been reported (5). Diabetes mellitus is also associated with deficiencies of vitamins and trace elements, particularly those of vitamin C (9–11), vitamin E (12) and zinc (5, 13, 14). The concentration of glucose is the most important physiological factor regulating the secretion of insulin in the blood flowing from pancreatic β cells.

The concentration of glucose in the blood influences both the secretion and the biosynthesis of that hormone. Simultaneously, insulin increases the activity of proteins transporting glucose (GLUT4) into the cell. This is connected with the glucose membrane receptor stimulation, which in turn affects the increase of the ATP amount in those cells. Numerous investigations of the activity of the vanadium complexes have shown promising results with regard to their potential use in medical care (15–17).

Vanadium is an element, which occurs in living organisms in trace amounts but it may still influ-

ence the metabolism of carbohydrates, lipids and cholesterol. Nevertheless, the exceeded level of this element is toxic. Anti-cancer/anti-carcinogen property of this element has also been noted (18).

Tolman et al. (19) demonstrated insulin-mimetic properties of the vanadium salts *in vitro*. This study has shown that various vanadium salts, similarly as insulin, stimulate the transportation and oxidation of glucose in adipocytes, increase the glycogen synthesis in the rat diaphragm and hepatocytes, and inhibit gluconeogenesis (GNG). The research conducted by Heyliger et al. (20) demonstrated that as a result of sodium vanadate treatment in the animal diabetes model, the normalization of the glucose level in the hyperglycemia conditions was achieved. It led to further research on the possibility of application of various vanadium compounds in the treatment of people with the Type 1 or Type 2 diabetes (21, 22).

Vanadium concentration in the blood of mammals is about 0.2–0.5 ng/mL, out of which nearly 80% is present in plasma, as a component of proteins such as transferrin and albumins (23). As the role of vanadium has not been explained yet, it seems justified to study the impact of vanadium compounds on oxidative stress.

Vanadium ions in body fluids are mainly present in the form of pentavalent metavanadium, while the compounds of this element at the fourth oxidation level show intra-cellular domination.

Vanadium ions enter the cell *via* the anion canals, where their reduction takes place, primarily in the presence of glutathione, catechol, cysteine, NADH, NADPH and L-ascorbic acid (24).

MATERIALS AND METHODS

Material and methods of the experiment were described in earlier publications (25, 26).

Animals and vanadium administration

Male Wistar rats weighing between 220–250 g were adapted to a 12 h/12 h day/night cycle, (day was from 8 a.m. to 8 p.m.) with humidity ranging between 75–85%. The animals were divided into 7 groups of 6 animals in each group. The animals from each group were housed in two cages (3 rats in each). After 3 days from the beginning of the experiment, 55 mg of streptozotocin in citric buffer (0.1 mol/L) solution per 1 kg of body mass was injected into the caudal vein in the volume of 1 mL/kg of body mass in all groups of animals. Three days subsequent to the injections, the level of glucose was measured using Exac Tech (Medisense) strip glu-

cometer. The glucose level measured in animal blood was higher than 17 mmol/L. After the measurement, the rats were separated into the following tested groups, diabetic control rats (D), diabetic rats treated with insulin (Di), 5 groups of diabetic rats treated with both insulin and tested complexes (Di 1–5). The water solutions of tested vanadium complexes were administered once a day before 10 a.m. by gavage in a dose of 50 $\mu\text{mol/kg}$ and 1 U/kg of insulin was injected subcutaneously. Five weeks after the beginning of the treatment, the rats were anesthetized (using thiopental 50 mg/kg) and then, the pancreas was collected. The organ was kept frozen in -20°C until the time of the analysis.

Synthesis of vanadium complexes

The complexes $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(2,2'\text{-bpy})] \times 8 \text{H}_2\text{O}$ (complex 1), $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(1,10'\text{-phen})] \times 5 \text{H}_2\text{O}$ (complex 2), $\text{Na}[\text{V}^{\text{VO}}(\text{O}_2)_2(4,4'\text{-Me-}2,2'\text{-bpy})] \times 8 \text{H}_2\text{O}$ (complex 3), $[\text{V}^{\text{IV}}\text{O}(\text{SO}_4)(1,10'\text{-phen})] \times 2 \text{H}_2\text{O}$, (complex 4), $[\text{V}^{\text{IV}}\text{O}(\text{SO}_4)(2,2'\text{-bpy})] \times \text{H}_2\text{O}$ (complex 5), where: 2,2'-bpy = 2,2'-bipyridine, 1,10'-phen = 1,10'-phenanthroline, 4,4'-Me-2,2'-bpy = 4,4'-dimethyl-2,2'-bipyridine were synthesized using methods described in the literature and their purity was confirmed by microanalysis and IR spectroscopy (27–29).

Pancreas tissue preparation

Frozen pancreas tissue was transferred directly to the lyophilizing cabinet and lyophilized using ABCONO FREEZONE 4,5. in the temperature from -40°C to -53°C , and under pressure of 14 Pa to 1 Pa. Then, the lyophilized organs were homogenized and pressed into pellets, about 1 mm thickness and 10 mm in diameter, under the pressure of 15 MPa. Such pellets were placed on Scotch tape and attached to an aluminum frame.

PIXE analysis

The PIXE (Proton Induced X-ray Emission) analysis was performed at the Institute of Nuclear Physics Polish Academy of Sciences (IFJ PAN) in Kraków (30). A multi-elemental probing was carried out using a 2 MeV proton beam (about 0.5 mm in diameter) from the Van de Graaff accelerator directed perpendicularly to the sample's surface. In order to get high quality X-ray characteristic spectra, the acquisition time for measuring each sample was set to 20 min. In every series 14 samples together with 2 standards (IAEA H-8 Horse Kidney and National Standards & Technology Standard Reference Material 1577b Bovine Liver) were mounted in the PIXE chamber. The standard materi-

als were used for energy calibration and determination of the trace elements' concentration levels. All the emitted X-ray quanta were detected with Si-Li detector with the energy resolution of 190 eV for the 5.9 keV line. The normalization was performed based on simultaneously detected spectra of back-scattered protons. Both the X-rays and back-scattered protons were recorded using Computer Automated Measurement and Control (CAMAC) electronic system. All the acquired spectra were analyzed with GupixWin ver. 2.0 software. All results, showing the level of metals' concentration in the rats' pancreas, are presented as 'box-and-whisker' plots showing median, lower and upper quartiles (box) and the farthest data (whiskers). The statistical calculations were performed using Statistica 7.1 program. Differences between the studied rat groups were estimated with the use of a non-parametric Kruskal-Wallis test that enables to compare three or more unpaired groups. Results with p level below 0.05 were considered statistically significant ($p < 0.05$).

RESULTS

Vanadium

The level of vanadium in pancreas of diabetic not-treated rats (D) and insulin-injected diabetic ones (Di) was in the range from 0.25 ± 0.06 to 0.33 ± 0.05 mg/kg of dry tissue. Similar data were reported by Frank et al. (31). In pancreas of vanadium compounds treated rats (Di1–Di5) the concentration of this element increased about 6 times as compared to the diabetic control groups D and about 4–5 times as compared to Di group, regardless of the chemical structure of the compound and oxidation state of vanadium (Fig. 1). This observation is similar to the data presented by Cremer et al. (32) where the rats were treated with an ^{48}V isotope complex. The total absorption of vanadium after oral administration of the tested complexes was not calculated, however, it is widely known that the level of V measured in intestinal system equals roughly to ten percent of an administered dose, and such a level of vanadium uptake was assumed in the presented study (33). The vanadium concentration in pancreas of vanadium-treated rats' ranged from 1.21 ± 0.27 to 1.69 ± 0.09 mg/kg of dry tissue. The lowest concentration growth of vanadium was observed in the pancreas of animals treated with complex 4, and the largest – with complex 5. Both of the compounds have the same vanadium oxidation state (IV) but different ligands. The changes of the vanadium content in pancreas of rats supplemented with compound 5

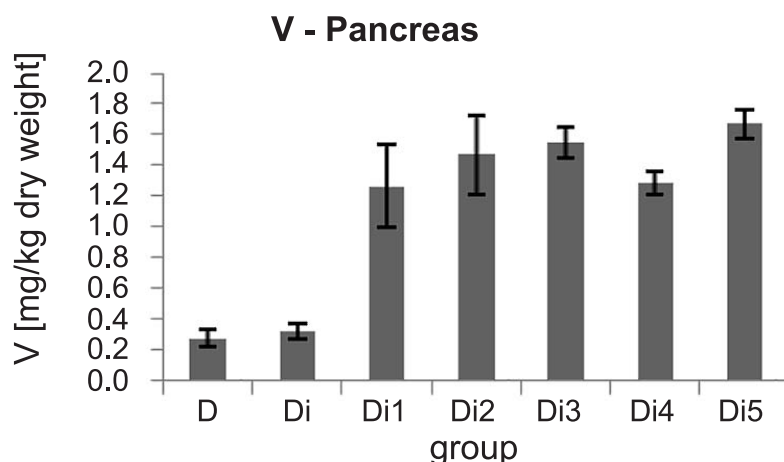


Figure 1. Vanadium level in the study rat groups: (group abbreviations: diabetic control rats (D), diabetic rats with insulin (Di), diabetic rats with insulin and tested complexes (Di 1 – $\text{Na}[\text{VO}(\text{O}_2)_2(2,2'\text{-bpy})] \times 8 \text{H}_2\text{O}$, Di2 – $\text{Na}[\text{VO}(\text{O}_2)_2(1,10'\text{-phen})] \times 5 \text{H}_2\text{O}$, Di3 – $\text{Na}[\text{VO}(\text{O}_2)_2(4,4'\text{-Me-2,2'}\text{-bpy})] \times 8 \text{H}_2\text{O}$, Di4 – $[\text{VO}(\text{SO}_4)(1,10'\text{-phen})] \times 2 \text{H}_2\text{O}$, Di5 – $[\text{VO}(\text{SO}_4)(2,2'\text{-bpy})] \times \text{H}_2\text{O}$)

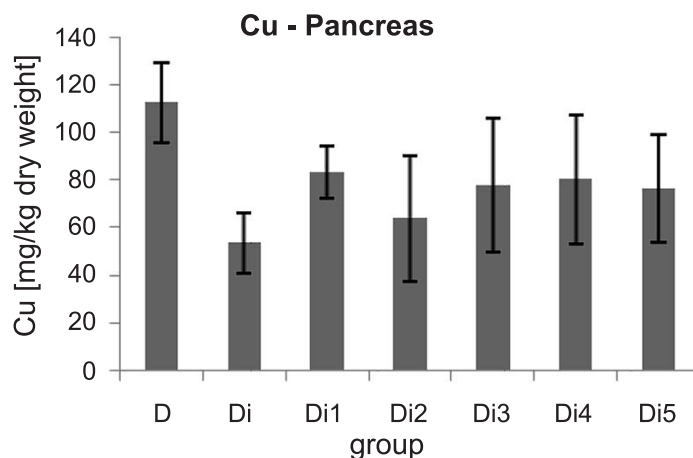


Figure 2. Copper level in animal groups: (group abbreviations see Figure 1)

were statistically significantly different in comparison to group D and Di ($p < 0.05$).

It is known that the presence of metal administration can influence the level of other metals such as iron, copper, zinc, manganese, calcium or potassium (34). Therefore, the concentrations of these metals in the pancreas have been determined in our analysis.

Copper

The contents of copper determined in pancreas for all the tested groups are presented in Fig. 2. The application of insulin (group Di) caused the largest decrease in the level of copper ($50.72 \pm 12.63 \text{ mg/kg}$ of dry tissue). Applying vanadium complexes

increased the Cu level in comparison to the Di group. Statistically significant growth of the concentration of this element occurred for the complex 1, including the vanadium (V) and ligands bipyridine (Di 1), and the complex 4 (Di 4) with vanadium (IV) and ligands phenyls (82.50 ± 11.03 and $81.34 \pm 26.95 \text{ mg/kg}$ of dry tissue, respectively).

Manganese

In case of the animals fed with the addition of vanadium complexes 1, 4 and 5, the concentration of manganese in the pancreas was reduced in comparison to the quantity of this microelement in the groups D and Di (Fig. 3). The twofold decrease of the manganese concentration was observed only in

case of the complex 1 ($p < 0.05$), while complexes 4 and 5 reduced the Mn quantity imperceptibly. The influence of complexes 2 and 3 on the Mn concentration in the pancreas was not observed.

Potassium

The concentration of potassium in the pancreas of the rats (Fig. 4), which were treated with vanadium complex 3 (16.32 ± 5.06 g/kg) increased in comparison to the animals from the group D (10.16 ± 3.27 g/kg) and Di (11.19 ± 2.17 g/kg), and the increase was statistically significant ($p < 0.05$). Organic complex of vanadium including methylbipyridine derivative (complex 3) raised the level of potassium in the pancreas in comparison to the complex including only bipyridine as a ligand (complex 1), and the result was statistically significant ($p < 0.05$).

The degree of the vanadium oxidation state essentially influenced the concentration of potassium in the pancreas. In case of vanadium complexes at the IV oxidation level (complex 4 and 5), a decrease in the potassium concentration in the pancreas was observed. The lowest potassium concentration occurred in group Di 4 (7.16 ± 1.88 g/kg). A decrease in potassium concentration in the pancreas in case of the Di 4 and Di5 groups in comparison to D and Di group, was observed but it was not statistically significant.

Iron

The highest concentration of the iron in the pancreas was observed in the diabetic control group (317.0 ± 112.9 mg/kg of dry tissue) (Fig. 5). After the injection of insulin in the Di group, the concen-

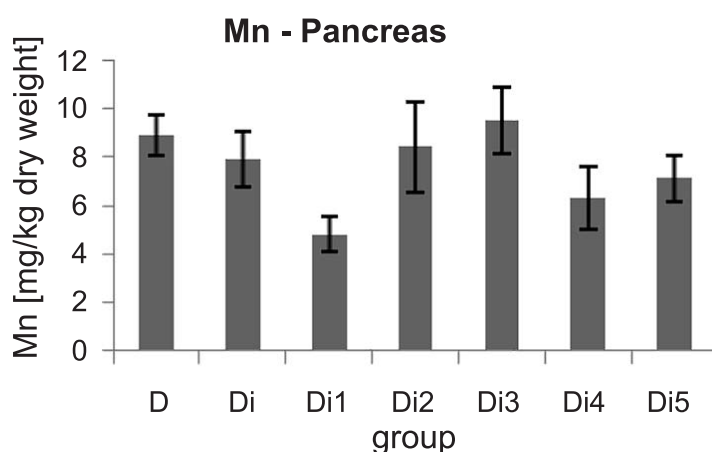


Figure 3. Manganese level in animal groups: (group abbreviations see Figure 1)

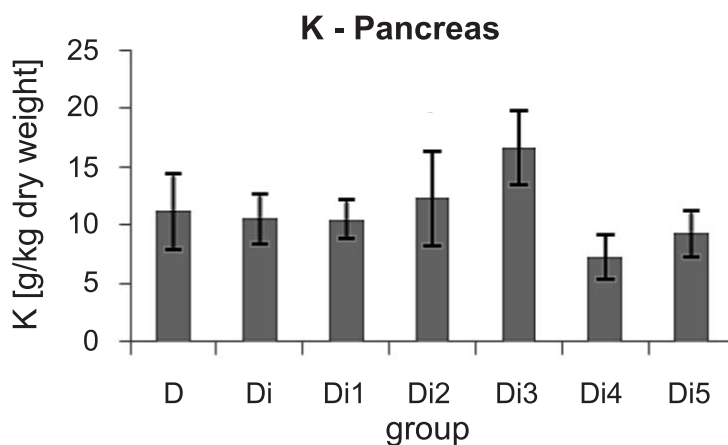


Figure 4. Potassium level in animal groups: (group abbreviations see Figure 1)

tration of this element dropped twice as compared to the D group. Vanadium complexes 1, 3, 4 and 5 reduced the concentration of Fe to a statistically significant degree in comparison to the groups Di ($p < 0.05$) and D ($p < 0.05$). It was also observed that complexes 2 and 3 had not influenced the change of the concentration of Fe in comparison to the Di group.

Zinc

As shown in Fig. 6, zinc concentration was the lowest in the D control group (19.83 ± 3.28 mg/kg), not treated with vanadium. In the diabetic group with insulin administration (Di), Zn concentration was also very low (22.06 ± 33.47 mg/kg). In all the vanadium-treated animal groups, the Zn concentration increased and the level was dependent on the type of vanadium complexes used. It was statistical-

ly significant and 2 up to 6 times higher in comparison to the diabetic group D and Di. Zn concentration was the highest in the pancreas of Di 1 and Di 2 animal groups (110.77 ± 13.74 mg/kg and 84.67 ± 16.34 mg/kg, respectively). In the Di 5 group, the increase of Zn concentration was the lowest for vanadium-treated rats (41.08 ± 17.28 mg/kg) but still about two times higher in comparison to the diabetic groups (D and Di), not treated with vanadium. After both vanadium compounds 3 and 4 administration similar zinc concentration in the pancreas (64.59 ± 8.66 mg/kg and 63.36 ± 8.42 mg/kg, respectively) was observed.

Calcium

As shown in Fig 7, calcium concentration in the pancreas was higher in the animals treated by the vanadium complexes 3 and 5 (Di 3 and Di 5, respec-

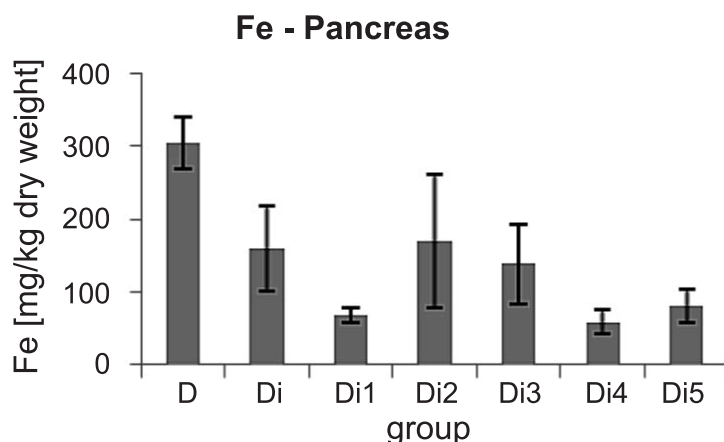


Figure 5. Iron level in the study animal groups: (group abbreviations see Figure 1)

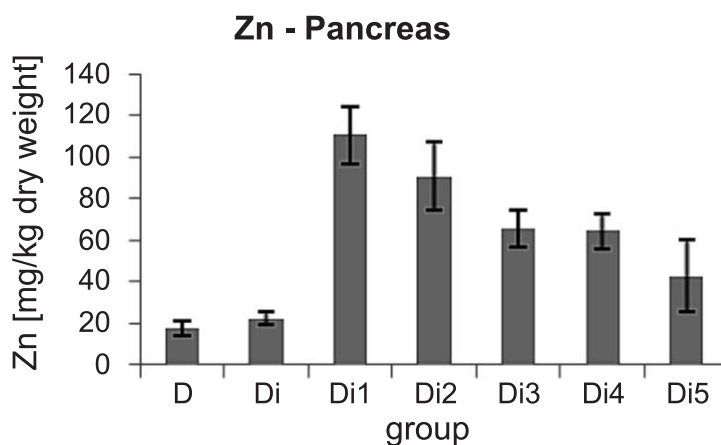


Figure 6. Zinc level in animal groups: (group abbreviations see Figure 1)

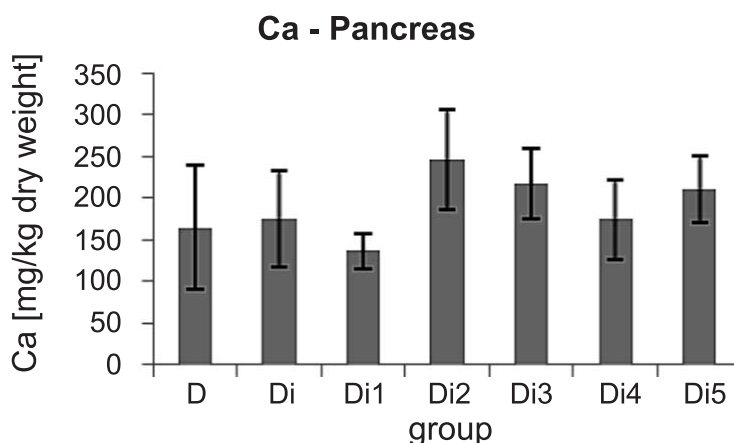


Figure 7. Calcium level in animal groups: (group abbreviations see Figure 1)

tively, 204.3 ± 177.8 mg/kg, $219.2 \pm 39/6$ mg/kg, 210.5 mg/kg) than in the D group of diabetic rats (172.3 ± 74.8 mg/kg), not treated with vanadium, and the Di group (168.3 ± 57.7 mg/kg). Vanadium (V) with bipyridine ligand (Di 1) treatment reduced calcium concentration in the pancreas in a statistically significant way in comparison to the effect of complex 3 and 5 and without statistical significance in comparison to the pancreas of not diabetic rats not treated with vanadium. Changes in the group Di 4 with vanadium (IV) with phenyl ligand in comparison to the diabetic not treated animals were not observed.

Complex 1 in comparison to the others vanadium complexes had the strongest influence on the Cu, Mn and Zn concentration and also an essential effect on Fe concentration in the pancreas and at the same time it had the weakest influence on the calcium, and potassium concentration. Complex 2 showed a strong influence on zinc concentration and mostly non influence on other elements' concentration as compared to the Di group, however a decrease in iron concentration as compared to the D group was visible. Complex 3 had a substantial influence on zinc and potassium concentration in the pancreas. All the three tested complexes (groups Di1–Di3) contained vanadium (V) and different ligands. The influence on the measured metal concentration in pancreas is associated not only with the vanadium oxidation state but also with the type of used ligands. Complexes 4 and 5 contained both vanadium (IV). Complex 4 caused more significant decrease on the potassium and iron concentrations while complex 5 on zinc concentration in the pancreas.

DISCUSSION

It has been shown that vanadium possesses anti-diabetic activity and that it can be used as a potential therapeutic agent in diabetes treatment in several diabetic models (23). Simultaneously, supplementation of this metal can influence the levels of other metals in tissues and also indirectly, metabolic parameters' such as enzymatic activity (35). Scientific investigations showed that vanadium has insulin mimetic properties and the ability of enhance the effect of insulin (22, 35). Moreover, vanadium can inhibit degradation of ligand-receptor complex in lysosomes in different types of cells (36).

Manganese is necessary for the metabolism of vitamin B1 and vitamin E. It activates some enzymes, takes part in the process of energy production, synthesis of glycogen as well as urea. During vanadium treatment, the changes of manganese concentration in rats' pancreas were not significant. This may suggest a minor influence of the tested vanadium complexes on the functions regulated by manganese in the pancreas. On the other hand, the ratio of manganese to other elements concentrations can affect the activity of some enzymes in pancreas. Therefore, the influence of vanadium on manganese level in pancreas and its possible effects should be further studied. Enzymes activated by manganese in pancreas and also possibly in other organs can be useful in the elimination of oxidative stress in patients with diabetes.

The increased potassium concentration may suggest hyperkalemia, which is usually the result of handicapped dismissing of potassium with urine and

excessive freeing of potassium from cells. This observation is associated only with the vanadium complex 3 with vanadium (V) and methylbipyridine ligand.

Iron is a component of organic biomolecules like: porphyrin, hemoglobin and myoglobin. It also occurs in active sites of some enzymes. The concentration of iron in the serum depends on absorption in the alimentary line. The highest concentration of iron is found in the intestines, the spleen (23) and the bone marrow. The decrease of the iron concentration causes, among others, a chronic failure of kidneys and/or the shortage of C vitamin absorption. In diabetic rats, not treated with insulin, the iron concentration was the highest. The administration of insulin decreases the concentration of that element in pancreas. This also confirms the insulin-mimetic action of vanadium in diabetes. The untreated diabetes and biochemical changes associated with this illness probably increase the iron concentration in pancreas tissue. This observation needs to be confirmed by additional investigation. Administration of insulin and/or vanadium caused the decrease of the copper level in pancreas. The tested vanadium complexes showed a similar influence on the level of iron in pancreas. However, the influence differed depending on the type of the vanadium complex used. In groups Di 1, Di 4 and Di 5 in comparison to the Di 2 and Di 3 the decrease of iron level was greater. Surprisingly, there's no clear trend of such influence. Complex 1 as compared to 4 and 5 differed in oxidation state (V vs. IV) while complex 4 differed in ligand environment as compared to complex 1 and 5. Additionally, the less significant decrease in Fe concentration in group Di2 and Di3 could be related to vanadium oxidation state (V) but this observation is in opposite to the influence of complex 1, which was also vanadium (V) component.

At the molecular and cellular level, zinc (Zn) is intimately involved in the insulin synthesis, secretion and signalling, and thus, in the subsequent effect of insulin on metabolism. Various clinical and epidemiological studies suggest that reduced Zn status is associated with diabetes (37, 38). The results of presented study support this observation and statement. The induced diabetes reduced zinc concentration in the pancreas of not treated animals.

Pancreas is the site of high Zn turnover and one of the few organs that show reduced Zn concentration during Zn deficiency (39). Reduced pancreatic Zn concentrations have been reported in genetic mouse models of Type 2 diabetes, ob/ob (mutation in ob (leptin) gene and db/db (mutation in leptin receptor)

mice (40–42) and GK rats (Type 2 model produced by selective breeding of rats with glucose intolerance) (43) as compared to the non-diabetic ones.

Although a lot of effort has been placed on studying Zn and pancreas, the consequences of reduced pancreatic concentrations of other minerals (e.g., Fe, Mn and Mg in GK rats) in co-action with Zn have not been explored (42). Adequate levels of pancreatic Zn may also be crucial to provide antioxidant protection, given that oxidative stress is a factor of tissue damage in Type 1 and Type 2 diabetes and it is associated with complications occurring in this disease (44, 45). Compared to several other tissues, β -cells have lower levels of antioxidant defense components and are susceptible to oxidative damage (46–48). Zinc contributes to antioxidant defense as a component of CuZn superoxide dismutase (CuZnSOD) and metallothionein (MT).

Calcium level in different investigated groups has not changed statistically and it is difficult to discuss the mutual relationship between the diabetes insulin and the tested vanadium complexes.

This investigation showed that diabetes and its' treatment has an influence on the elemental level measured in pancreas. Pancreas, being very important organ in the diabetes control, is responsible for the glucose level changes by insulin secretion response. The investigated changes in the trace elements' level were mostly perceptible for zinc and iron. Zinc plays an important role in the physiological function of pancreas. The animal group with diabetes induced by STZ had significantly lowered zinc level in comparison to the vanadium-treated diabetic groups. Vanadium stimulates the activity of adenylate cyclase (CA), phospholipase C (PLC) and phospholipase A₂ (PLA₂) (47). This action results in the increased concentration of the secondary transmitters, such as cAMP, IP₃, DAG and also arachidic acid. The increase of IP₃ and arachidic acid level caused an increase of the level of Ca²⁺ ions. It is possible that all these mechanisms participate also in the zinc level elevation in pancreas. All the effects associated with an anti-diabetic activity of vanadium are not only the results of the insulin mimetic signalling pathway but also of the zinc concentration protection, especially in pancreas. Zinc is an element, which is known to have a protective activity in the Type 2 diabetes. Such an observation is reported for the first time in the literature. Therefore, it is not possible to discuss it with the results of other researchers.

Currently, the mutual interaction between vanadium and zinc in pancreas remains unknown. Better understanding of their interaction may offer

another possibility of increasing the understanding of diabetes mechanisms and possible procedures for treatment of that disease.

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DRUG SYNTHESIS

SYNTHESIS OF SOME NEW CARBONITRILES AND PYRAZOLE COUMARIN DERIVATIVES WITH POTENT ANTITUMOR AND ANTIMICROBIAL ACTIVITIES

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Abstract: 3-Acetyl-4-hydroxycoumarin (**2**) was reacted with some aldehydes (4-chlorobenzaldehyde, 4-bromobenzaldehyde, 5-methylfurfural) to afford the chalcones (**3a–c**). Cyclization of these chalcones with malonitrile in the presence of ammonium acetate afforded pyridine carbonitriles (**4a–c**), while the cyclization reaction of chalcones (**3a–c**) with ethyl cyanoacetate afforded the oxypyridine carbonitriles (**5a–c**). On the other hand, the chalcones (**3a–c**) reacted with hydrazine hydrate in alcohol to yield pyrazoles (**6a–c**), but when the same reaction is carried out in the presence of acetic acid, the acetyl pyrazole derivatives (**7a–c**) were obtained. Finally, the reaction of the chalcones (**3a–c**) with phenylhydrazine afforded phenylpyrazole derivatives (**8a–c**). The structures of synthesized compounds were confirmed by their micro analysis and spectral data (IR, NMR and MS). Twelve samples were evaluated for the human breast adenocarcinoma cytotoxicity, three of them showed moderate activity, the rest of the samples showed weak cytotoxic activity (very high IC₅₀), but for the hepatocarcinoma cell lines four samples showed weak cytotoxic effect, while the rest of the compounds showed very weak effect. For antimicrobial study, three compounds proved to be the most promising against tested bacterial organisms.

Keywords: coumarin, chalcones, carbonitriles, pyrazoles, antitumor, antimicrobial

Cancer represents one of the most severe health problems worldwide and the development of new anticancer drugs and more effective strategies are the areas of the utmost importance in drug discoveries and clinical therapy. Much of the research in these areas is currently focused in cancer specific mechanism and the corresponding molecular targets (1).

Coumarins are classified as a members of benzopyrone family. All of them consist of a benzene ring joined to a pyrone ring. Coumarins are of great interest due to their pharmacological properties, in particular, their antitumor and antimicrobial activity made these compounds attractive for further derivatization and screening as novel therapeutic agents. The literature investigation revealed cytotoxic activity of coumarin against several human tumor cell lines (2). From the literature, we can conclude that the chalcones have been reported to possess biolog-

ical property as antitumor activity (3–6) and antimicrobial activity (7).

It was found that pyrazoles present an interest group of compounds, many of which possess widespread pharmacological properties as antitumor activities (8–11) and antimicrobial activity (12). In addition, we can say that the anticancer activity of many compounds is due to the presence of nitrogen heterocyclic ring (13).

Our goal in this work was to prepare some carbonitriles and pyrazolyl 4-hydroxycoumarins with potent antitumor and antimicrobial activities.

EXPERIMENTAL

Materials and methods

Melting points were determined on Electrothermal IA 9000 apparatus and were uncorrected. Elemental microanalyses were performed on

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Elementar, Vario EL, at the microanalytical center. The infrared (IR) spectra were recorded on Nexus 670 FT-IR FT-Raman spectrometer in potassium bromide discs, the proton nuclear magnetic resonance ($^1\text{H NMR}$) spectra were determined on Varian Mercury 500 MHz spectrometer, using tetramethylsilane (TMS) as an internal standard. The mass spectra (MS) were performed on Jeol JMS-AX500 mass spectrometer. All spectral data were carried out at the National Research Center, Cairo, Egypt. The reactions were followed by TLC (silica gel, aluminum sheets 60 F 254, Merck) using benzene : ethyl acetate (8 : 2, v/v) as eluent and visualized with iodine-potassium iodide reagent.

Synthesis of 3-acetyl-4-hydroxy-2H-chromen-2-one (2)

3-Acetyl-4-hydroxycoumarin has been synthesized by boiling 4-hydroxycoumarin (**1**) (1 g, 6 mmol) with phosphorus oxychloride (2 mL) in glacial acetic acid (5 mL). The solution was cooled and water was added to precipitate the desired yellowish brown solid of 3-acetyl-4-hydroxycoumarin (0.90 g, 73% yield) that was recrystallized from ethyl alcohol – water to yield the desired compound with m.p. 132–134°C (as reported in (14)).

General procedure for the synthesis of 1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-3-aryl-2-propen-1-ones (3a–c)

A solution of 3-acetyl-4-hydroxycoumarin (**2**) (1 g, 5 mmol) in ethyl alcohol (10 mL) and the selected aldehyde, namely: 4-bromobenzaldehyde, 4-chlorobenzaldehyde, and 5-methylfurfural (5 mmol) in the presence of piperidine (1 mL) was refluxed for 5–7 h. The solution was cooled and water was added to precipitate the desired chalcone compound.

Compounds **3a** and **3b** were prepared according to Završink et al. (15).

4-Hydroxy-3-[(E)-3-(5-methylfuran-2-yl)acryloyl]-2H-chromen-2-one (3c)

M.p. 218–221°C; yield 84%. IR (v, cm^{-1}): 3434 (OH, brs), 2974 (-CH aliphatic stretching), 1725 (C=O, α -pyrone), 1611 (C=O), 1527 (C=C). $^1\text{H NMR}$ (CDCl_3 , δ , ppm): 2.2 (3H, s, CH_3), 6.2–6.8 (2H, d, acryl-H), 7.2–8.1 (6H, m, Ar-H), 9.1 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+) 296 (15%), 279 (32%), 254 (20%), 135 (3%), 64 (100%).

General procedure for the synthesis of 4-aryl-2-amino-6-(4-hydroxy-2-oxo-2H-chromen-3-yl)-pyridine-3-carbonitriles (4a–c)

Method A: To a mixture of 3-acetyl-4-hydroxycoumarin (1 g, 5 mmol) and the appropriate aldehyde, namely: 4-bromobenzaldehyde, 4-chlorobenzaldehyde or 5-methylfurfural (5 mmol) in ethyl alcohol (20 mL), malononitrile (0.33 g, 5 mmol) and ammonium acetate (0.75 g, 10 mmol) were added. The reaction mixture was refluxed for 5–6 h. The obtained solid was filtered off, washed with absolute ethyl alcohol and recrystallized from methyl alcohol to give the desired compounds.

Method B: An ethanolic mixture of the selected chalcone (**3a–c**) (1.25 mmol), and malononitrile (0.06 g, 1.25 mmol) in the presence of ammonium acetate (0.2 g, 2.5 mmol) was refluxed for 4–5 h. After cooling, the obtained solid was filtered off, washed with ethyl alcohol and recrystallized from methyl alcohol to give the title compounds.

Compounds **4b** and **4c** were prepared according to Mohamed et al. (16).

2-Amino-4-(4-bromophenyl)-6-(4-hydroxy-2-oxo-2H-chromen-3-yl)pyridine-3-carbonitrile (4a)

M.p. 295–295°C; yield: 89%. IR (v, cm^{-1}): 3472 (OH, brs), 3372 (NH_2), 2213 (C=N), 1698 (C=O, α -pyrone), 1595 (C=N). $^1\text{H NMR}$ (DMSO-d_6 , δ , ppm): 4.33 (2H, s, NH_2 , D_2O exchangeable), 7.55–8.5 (8H, m, Ar-H) and 7.33 (1H, s, pyridine proton), 8.92 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): ($\text{M}^+ + 2$) 436 (35%), 434 (100%), 408 (13%), 313 (22%), 179 (13%), 121 (93%).

General procedure for the synthesis of 4-aryl-1,2-dihydro-6-(4-hydroxy-2-oxo-2H-chromen-3-yl)-2-oxopyridine-3-carbonitriles (5a–c)

An ethanolic mixture of chalcone (**3a–c**) (1.6 mmol), and ethyl cyanoacetate (0.19 mL, 1.6 mmol) in the presence of ammonium acetate (0.23 g, 3.33 mmol) was refluxed for 12 h. After cooling, the obtained solid was filtered off, washed with ethyl alcohol and recrystallized from methyl alcohol to give the title compounds.

4-(4-Chlorophenyl)-1,2-dihydro-6-(4-hydroxy-2-oxo-2H-chromen-3-yl)-2-oxopyridine-3-carbonitrile (5b)

M.p. 171–173°C; yield 80%. IR (v, cm^{-1}): 3420 (OH, brs), 3100 (NH), 2700 (C=N), 1680 (C=O, α -pyrone), 1612 (C=O). $^1\text{H NMR}$ (DMSO-d_6 , δ , ppm): 7.9 (1H, s, NH, D_2O exchangeable), 7.2–7.8 (8H, m, Ar-H) and 6.8 (1H, s, pyridine carbonitrile proton), 14.6 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+) 390 (22%), 364 (36%), 312 (65%), 229 (8%), 163 (13%).

1,2-Dihydro-6-(4-hydroxy-2-oxo-2H-chromen-3-yl)-4-(5-methylfuran-2-yl)-2-oxopyridine-3-carbonitrile (5c)

M.p. 100–102°C; yield 70%. IR (ν , cm^{-1}): 3228 (OH, brs) 3124 (NH), 2272 (C=N), 1746 (C=O, α -pyrone), 1667 (C=O). ^1H NMR (DMSO- d_6 , δ , ppm): 2.4 (3H, s, CH_3), 7.25 (1H, s, NH, D_2O exchangeable), 7.3–8.1 (6H, m, Ar-H) and 6.5 (1H, s, pyridine carbonitrile proton), 14.5 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+) 360 (0.56%), 337 (0.45%), 257 (0.71%), 151 (8%), 109 (21%), 63 (22%).

General procedure for the synthesis of 3-(5-aryl-4,5-dihydro-1H-pyrazol-3-yl)-4-hydroxy-2H-chromen-2-one derivatives (6a–c)

A mixture of the appropriate chalcone (**3a–c**) (1 mmol) and hydrazine hydrate 99% (2 mmol) in ethyl alcohol (30 mL) was refluxed for one hour. The reaction mixture was cooled and the formed precipitate was filtered off, washed and recrystallized from methyl alcohol to give compounds (**6a–c**).

3-[5-(4-Bromophenyl)-4,5-dihydro-1H-pyrazol-3-yl]-4-hydroxy-2H-chromen-2-one (6a)

M.p. 229–231°C; yield 84%. IR (ν , cm^{-1}): 3289 (OH, brs), 3167 (NH), 1675 (C=O, α -pyrone), 1608 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 3.5 (1H, dd, H_a), 4.05 (1H, H_c), 4.8 (1H, dd, H_b), 7.0 (1H, s, NH, D_2O exchangeable), 7.2–8.0 (8H, m, Ar-H), 13.37 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+ + 2) 386 (2%), 384 (7%), 303 (15%), 226 (41%), 78 (9%).

3-[4,5-Dihydro-5-(5-methylfuran-2-yl)-1H-pyrazol-3-yl]-4-hydroxy-2H-chromen-2-one (6c)

M.p. 228–230°C; yield 90%. IR (ν , cm^{-1}): 3746 (OH, brs), 3220 (NH), 1673 (C=O, α -pyrone), 1614 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 2.3 (3H, s, CH_3), 3.9 (1H, dd, H_a), 5.8 (1H, H_c), 6.3 (1H, dd, H_b), 7.2–8.0 (6H, m, Ar-H), 7.0 (1H, s, NH, D_2O exchangeable), 13.37 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+) 310 (9%), 295 (12%), 229 (5%), 201 (20%), 81 (5%).

General procedure for the synthesis of 3-(5-aryl-1-acetyl-4,5-dihydro-1H-pyrazol-3-yl)-4-hydroxy-2H-chromen-2-ones (7a–c)

A mixture of chalcone (**3a–c**) (1 mmol) and hydrazine hydrate 99% (2 mmol) in glacial acetic acid (30 mL) was refluxed for 4–8 h. The reaction mixture was cooled and diluted with water, the formed precipitate was filtered off, washed and

recrystallized from ethyl alcohol to give compounds (**7a–c**).

3-[1-Acetyl-5-(4-bromophenyl)-4,5-dihydro-1H-pyrazol-3-yl]-4-hydroxy-2H-chromen-2-one (7a)

M.p. 261–263°C; yield 78%. IR (ν , cm^{-1}): 3424 (OH, brs), 2950 (-CH aliphatic stretching), 1723 (C=O, α -pyrone), 1667 (C=O), 1615 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 2.4 (3H, s, COCH_3), 3.6 (1H, dd, H_a), 4.1 (1H, H_c), 5.4 (1H, dd, H_b), 7.1–8.02 (8H, m, Ar-H), 13.37 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+ + 2) 429 (2%), 427 (5%), 337 (16%), 287 (32%), 234 (51%), 220 (37%), 116 (30%).

3-[1-Acetyl-5-(4-chlorophenyl)-4,5-dihydro-1H-pyrazol-3-yl]-4-hydroxy-2H-chromen-2-one (7b)

M.p. 251–254°C; yield 85%. IR (ν , cm^{-1}): 3425 (OH, brs), 2952 (-CH aliphatic stretching), 1718 (C=O, α -pyrone), 1668 (C=O), 1616 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 2.39 (3H, s, COCH_3), 3.6 (1H, dd, H_a), 4.09 (1H, H_c), 5.51 (1H, dd, H_b), 7.1–8.02 (8H, m, Ar-H), 13.37 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+) 382 (1%), 360 (23%), 277 (27%), 245 (6%), 230 (100%), 171 (8%), 111 (20%).

General procedure for the synthesis of 3-(5-aryl-4,5-dihydro-1-phenyl-1H-pyrazol-3-yl)-4-hydroxy-2H-chromen-2-one derivatives (8a–c)

A mixture of the selected chalcone (**3a–c**) (1 mmol) and phenylhydrazine (0.1 mL, 1 mmol) in ethyl alcohol (30 mL) was refluxed for 1–2 h. The reaction mixture was cooled and the formed precipitate was filtered off, washed and recrystallized from methyl alcohol to give the desired compounds (**8a–c**).

3-(5-(4-Bromophenyl)-4,5-dihydro-1-phenyl-1H-pyrazol-3-yl)-4-hydroxy-2H-chromen-2-one (8a)

M.p. 227–229°C; yield 87%. IR (ν , cm^{-1}): 3444 (OH, brs), 1715 (C=O, α -pyrone), 1616 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 3.5 (1H, dd, H_a), 4.2 (1H, H_c), 5.17 (1H, dd, H_b), 6.8–8.04 (13H, m, Ar-H), 13.9 (1H, s, OH, D_2O exchangeable). MS: m/z (R.A. %): (M^+ + 2) 463 (5%), 461 (3%), 443 (3%), 383 (5%), 305 (8%), 213 (10%), 168 (64%).

3-[4,5-Dihydro-5-(5-methylfuran-2-yl)-1-phenyl-1H-pyrazol-3-yl]-4-hydroxy-2H-chromen-2-one (8c)

M.p. 201–203°C; yield 92%. IR (ν , cm^{-1}): 3421 (OH, brs), 1710 (C=O, α -pyrone), 1593 (C=N). ^1H NMR (CDCl_3 , δ , ppm): 2.2 (3H, s, CH_3), 3.83 (1H,

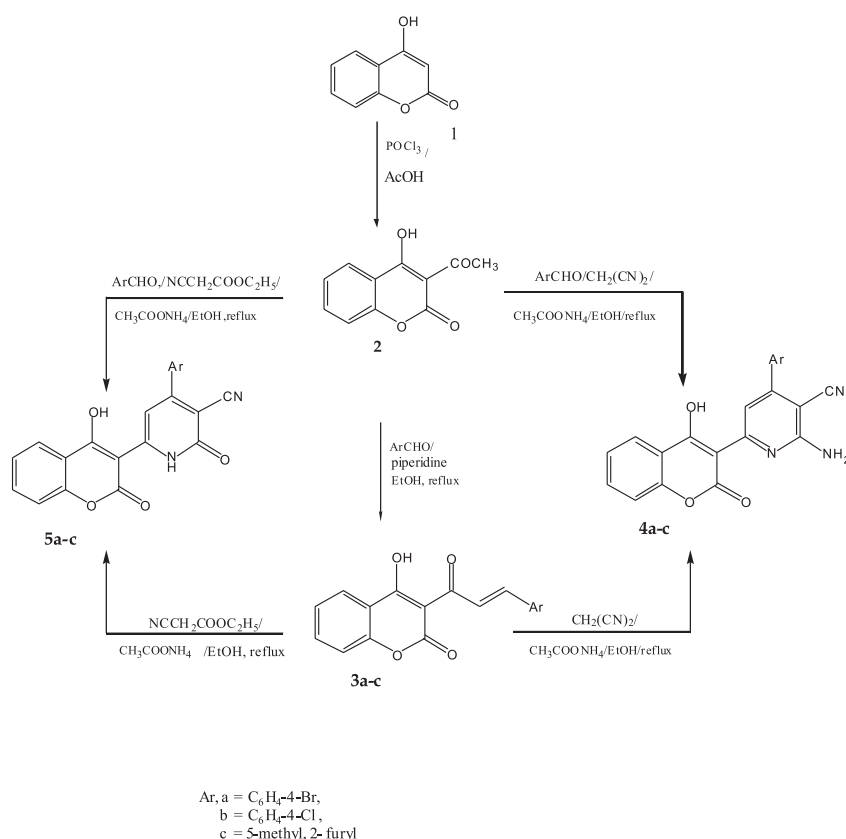
dd, H_a), 4.1 (1H, H_c), 5.1 (1H, dd, H_b), 5.8–8.03 (11H, m, Ar-H), 13.9 (1H, s, OH, D₂O exchangeable). MS: m/z (R.A. %): (M⁺) 386 (3%), 369 (10%), 309 (5%), 285 (7%), 161 (13%), 77 (81%).

Antitumor activity

Cell culture

Human hepatocarcinoma cell line (HepG2) and human breast adenocarcinoma cell line (MCF-7) purchased from ATCC (American Type Culture Collection), were used to evaluate the cytotoxic effect of the tested samples. Cells were routinely cultured in DMEM (Dulbecco's modified Eagle's medium), which was supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, containing 100 units/mL of penicillin G sodium, 100 units/mL of streptomycin sulfate, and 250 ng/mL of amphotericin B. Cells were maintained at sub-confluency at 37°C in humidified air containing 5% CO₂. For sub-culturing, monolayer cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when confluence had reached 75%. Tested samples

(20 μL) were dissolved in dimethyl sulfoxide (DMSO), and then diluted serially in the assay to begin with the mentioned concentration. All cell culture material was obtained from Cambrex BioScience (Copenhagen, Denmark). All chemicals were from Sigma/Aldrich, USA, except mentioned otherwise. All experiments were repeated three times, unless mentioned otherwise. Cytotoxicity of tested samples was measured against HepG2 cells using the MTT Cell Viability Assay. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals, which are largely impermeable to cell membranes, resulting in its accumulation within healthy cells. Solubilization of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm (17).



Scheme 1. Synthesis of 4-hydroxycoumarin-3-yl chalcones (3a-c) and 4-hydroxycoumarin-3-ylpyridine carbonitrile derivatives (4a-c) and (5a-c)

Table 1. Microanalysis of the synthesized derivatives.

Comp. no.	Molecular formula	Molecular weight	Microanalysis %	
			Calcd.	Found
3c	C ₁₇ H ₁₂ O ₅	296	C 68.92 H 4.08	C 68.89 H 4.1
4a	C ₂₁ H ₁₂ BrN ₃ O ₃	434.24	C 58.08 H 2.79 N 9.68	C 58.00 H 2.75 N 9.63
5a	C ₂₁ H ₁₁ BrN ₂ O ₄	435.2	C 57.59 H 2.55 N 6.44	C 57.56 H 2.57 N 6.33
5b	C ₂₀ H ₁₂ ClN ₂ O ₄	390.7	C 64.54 H 2.84 N 7.17	C 64.49 H 2.80 N 7.09
5c	C ₂₀ H ₁₂ N ₂ O ₅	360.32	C 66.67 H 3.36 N 7.77	C 66.69 H 3.29 N 7.70
6a	C ₁₈ H ₁₃ Br N ₂ O ₃	384	C,57.59 H,2.55 N,6.44	C 57.61 H 2.49 N 6.41
6b	C ₁₈ H ₁₃ Cl N ₂ O ₃	340.7	C 63.44 H 3.85 N 8.22	C 63.40 H 3.81 N 8.25
6c	C ₁₇ H ₁₄ N ₂ O ₄	310.3	C 65.80 H 4.55 N 9.03	C 65.78 H 4.51 N 8.97
7a	C ₂₀ H ₁₅ Br N ₂ O ₄	427	C 56.22 H 3.54 N 6.56	C 56.19 H 3.57 N 6.51
7b	C ₂₀ H ₁₅ Cl N ₂ O ₄	382.8	C 62.75 H 3.95 N 7.32	C 62.71 H 3.97 N 7.29
7c	C ₁₉ H ₁₆ N ₂ O ₅	352.3	C 64.77 H 4.58 N 7.95	C 64.72 H 4.62 N 7.90
8a	C ₂₄ H ₁₇ Br N ₂ O ₃	461.3	C 62.49 H 3.71 N 6.07	C 62.51 H 3.69 N 6.00
8b	C ₂₄ H ₁₇ Cl N ₂ O ₃	416	C 69.15 H 4.11 N 6.72	C 69.11 H 4.09 N 6.69
8c	C ₂₃ H ₁₈ N ₂ O ₄	386.4	C 71.49 H 4.70 N 7.25	C 71.45 H 4.68 N 7.27

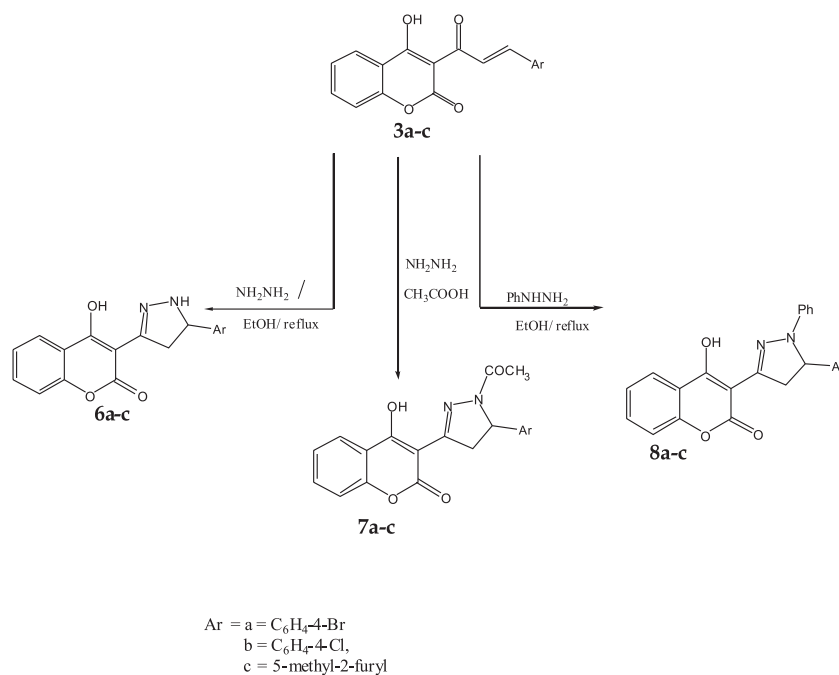
Reagents preparation

MTT solution: 5 mg/mL of MTT in 0.9% NaCl. Acidified isopropanol: 0.04 M HCl in absolute isopropanol.

Procedure

Cells (0.5×10^5 cells/well), in serum-free media, were plated in a flat bottom 96-well microplate, and treated with 20 μ L of different con-

centrations of the tested sample for 48 h at 37°C, in a humidified 5% CO₂ atmosphere. After incubation, media were removed and 40 μ L MTT solution/well were added and incubated for additional 4 h. MTT crystals were solubilized by adding 180 μ L of acidified isopropanol/well and plate was shaken at room temperature, followed by photometric determination of the absorbance at 570 nm using microplate ELISA reader. Triplicate repeats were performed for



Scheme 2. Synthesis of 4-hydroxycoumarin-3-yl-pyrazoline derivatives

Table 2. Antimicrobial activity of the synthesized compounds.

Test organisms → ↓ Comp. no.	Diameter of inhibition zone (mm)						
	Bacterial strain			Yeast	Fungal strain		
	<i>B. subtilis</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>	<i>F. oxysporum</i>	<i>F. solani</i>	<i>F. verticillioides</i>
2	15	20	25	18	12	18	12
3a	16	14	15	11	–	12	–
3b	16	17	15	12	–	–	–
4b	16	–	12	–	–	–	–
4c	11	13	17	10	16	12	14
5a	10	–	14	–	–	–	–
5c	30	26	24	25	12	14	11
8b	17	–	–	–	15	–	–

– = no inhibition for a microbe by the tested compound

each concentration and the average was calculated. Data were expressed as the percentage of relative viability compared with the untreated cells compared with the vehicle control, with cytotoxicity indicated by < 100% relative viability.

Calculation

Percentage of relative viability was calculated using the following equation: [Absorbance of treat-

ed cells/Absorbance of control cells) × 100, then the half maximal inhibitory concentration (IC₅₀) was calculated from the equation of the dose response curve.

Antimicrobial testing

Media

Czapek-Dox agar (CDA) (20): NaNO₃ 2.0 g; K₂HPO₄ 1.0 g; KCl 0.5 g; MgSO₄ × 7 H₂O 0.5 g;

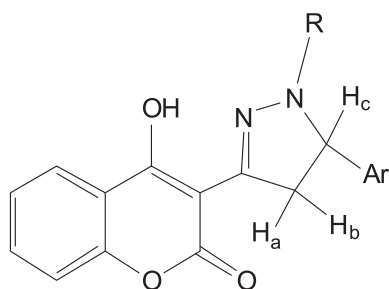


Figure 1. A general model for proton positions in the pyrazole ring of compounds (6a–c) – (8a–c)

FeSO₄ × 7 H₂O 0.001 g; sucrose 30 g; agar 20 g; H₂O 1 L.

Nutrient agar (NA): mass/volume): 0.5% peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl and 1000 mL distilled water, pH adjusted to neutral (6.8) at 25°C.

Antagonistic effect between compounds and test organisms

The aim of these experiments was to determine the antimicrobial activities of the selected compound against pathogenic fungi (*Fusarium oxysporum*, *Fusarium solani* and *Fusarium verticillioides*), bacteria (*Bacillus subtilis*; *Escherichia coli* and *Staphylococcus aureus*) and yeast (*Candida albicans*).

All the synthesized compounds were screened for their toxicity against the pathogenic fungi, bacteria and yeast. Two mL spore suspension of 7 days old culture of fungi, bacteria and yeast were inoculated on surface of Petri dish containing CDA medium for fungi and NA medium for bacteria and yeast. Filter paper disk method was applied (18, 19). A sample of 20 µg of the pure toxin was dissolved in the proper solvent (CHCl₃, DMSO) and applied to the filter paper disk (5 mm in diameter). The prepared disks were dried and firmly applied to the surface of the inoculated agar plates, then the plates were incubated at 28–30°C for 48–72 h for fungi and for 24 h for bacteria. Diameter of inhibition zone around each disk was measured in mm.

RESULTS AND DISCUSSION

Chemistry

The present work deals with the synthesis of some pyridine carbonitriles and pyrazolyl derivatives derived from the 4-hydroxycoumarin of expected antitumor and antimicrobial activities.

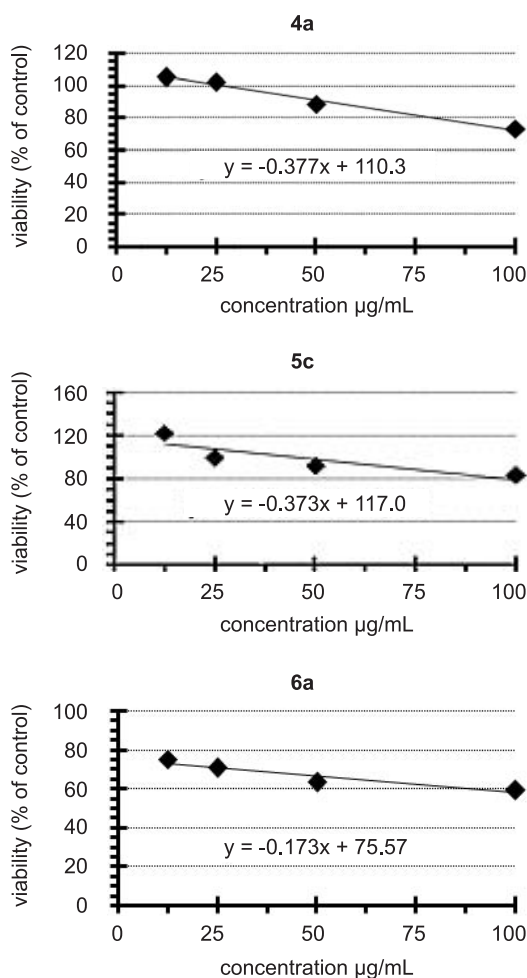


Figure 2. Cytotoxic effect of different samples against MCF-7 cells using MTT assay (n = 4); data expressed as the mean value of cell viability (% of control) ± SE

The reflux of 4-hydroxycoumarin (1) with acetic acid in the presence of phosphorus oxychloride leads to the formation of 3-acetyl-4-hydroxycoumarin (2), which reacted with some aldehydes (4-chlorobenzaldehyde, 4-bromobenzaldehyde, 5-methylfurfural) to yield the chalcones (3a–c) (Scheme 1).

The cyclization of the chalcones (3a–c) with malonitrile in ethyl alcohol in the presence of ammonium acetate leads to the formation of pyridine carbonitrile, (4a–c) (Scheme 1), while the reaction of (3a–c) with ethyl cyanoacetate afforded the oxopyridine carbonitrile (5a–c) (Scheme 1).

The pyrazolyl derivatives (6a–c) (Scheme 2) were formed by the reaction of the chalcones (3a–c) with hydrazine hydrate in alcohol, while the acetylpyrazole derivatives (7a–c) (Scheme 2) were formed when the same reaction is carried out in the

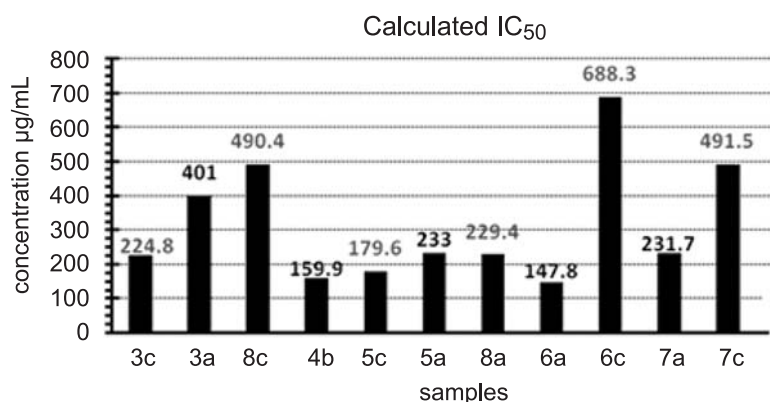


Figure 3. Calculated IC₅₀ for the tested samples indicating difference in toxicity between samples. (IC₅₀ = dose of the compound which reduces survival by 50%)

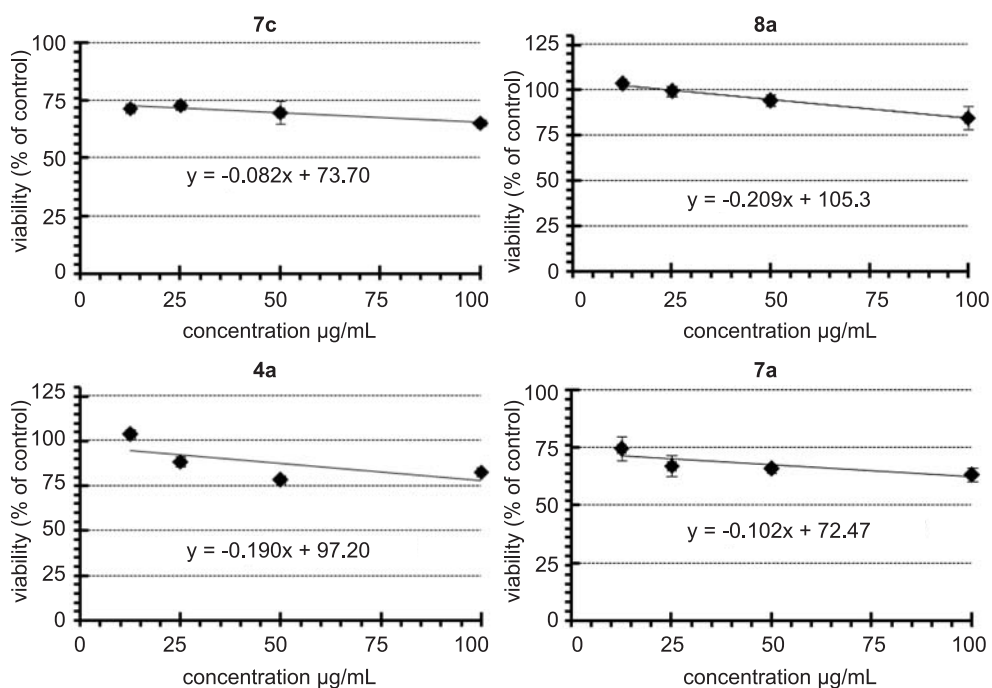


Figure 4. Cytotoxic effect of different samples against Hep-G2 cells using MTT assay ($n = 4$); data expressed as the mean value of cell viability (% of control) \pm SE

presence of acetic acid; on the other hand, the reaction of the chalcones (**3a–c**) with phenylhydrazine resulted in the formation of phenylpyrazole derivatives (**8a–c**) (Scheme 2).

The structures of the new synthesized compounds were confirmed by spectral data (IR, NMR and MS). The physicochemical characteristics are presented in Table 1.

Antitumor activity

Cytotoxic activity for 12 synthesized compounds were tested against human breast adenocarcinoma and the hepatocarcinoma cell lines.

The effect of the samples on the proliferation of MCF-7 cells was studied after 48 h of incubation. The treatment with **4a**, **5c** and **6a** showed almost moderate cytotoxic effect against MCF-7, as con-

cluded from their close IC_{50} values 159.9, 179.6 and 147.8 $\mu\text{g/mL}$, respectively, as shown in Figure 2, treatment with samples **3c**, **5a**, **8a** and **7a** showed weak cytotoxic effect as they had higher IC_{50} calculated to be 224.8, 233, 229.4 and 231.7 $\mu\text{g/mL}$, respectively. Samples **3a**, **8c**, **6c** and **7c** showed very weak cytotoxic effect concluded from their very high IC_{50} 401, 490.4, 688.3 and 491.5 $\mu\text{g/mL}$, respectively. Finally, sample **4c** did not show any cytotoxic effect as it has very high IC_{50} (2654 $\mu\text{g/mL}$) as shown in Figure 3. For human hepatocarcinoma cell line (HepG2), samples **3c**, **3a**, **8c**, **4c**, **5a**, **6a**, **6c** and **5c** did not show any cytotoxic effect as they increased proliferation of cells and samples **4a**, **8a**, **7a** and **7c** showed weak cytotoxic effect as shown in Figure 4.

Antimicrobial activity

The data in Table 2 show various degrees of antagonism against pathogenic bacteria, yeast and fungi. Three compounds proved to be most promising against tested bacterial organisms i.e., compounds **2**, **4c** and **5c**, which were the most active against both Gram negative (*Escherichia coli*) and Gram positive bacteria (*Bacillus subtilis*, and *Staphylococcus aureus*). Compounds **3b** and **3a** proved to be active against bacteria and yeast while no activity was determined with fungi. Compounds **2** and **5c** proved to be most active against *Candida albicans* and all tested fungal organisms. Compounds **4b**, **5a** and **8b** showed moderate activity and the rest of the compounds showed low effect against the tested bacterial organisms.

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SYNTHESIS AND MOLECULAR DOCKING OF SOME NOVEL ANTICANCER SULFONAMIDES CARRYING A BIOLOGICALLY ACTIVE PYRROLE AND PYRROLOPYRIMIDINE MOIETIES

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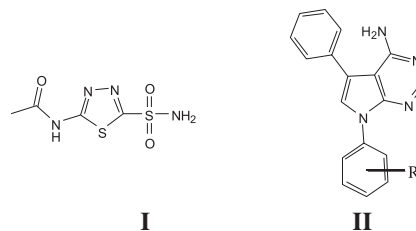
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Abstract: A novel series of pyrroles and pyrrolopyrimidines carrying a biologically active sulfonamide moiety have been synthesized. The structures were confirmed by elemental analyses and spectral data. All the target compounds were subjected to *in vitro* cytotoxic screening on breast cancer cell line (MCF-7). Most of the synthesized compounds showed good activity as cytotoxic agents with better IC₅₀ than doxorubicin as a reference drug. In order to suggest a mechanism of action for their activity, molecular docking on the active site of human c-Src was performed for all synthesized compounds.

Keywords: sulfonamide derivatives, anticancer activity

Nitrogen heterocyclic compounds are of special interest because they constitute an important class of natural and synthetic products, many of which exhibit useful biological activities. Pyrrole and fused pyrrole compounds such as pyrrolopyrimidine derivatives exhibit a broad spectrum of biological activities such as antimicrobial (1), analgesic (2), anti-inflammatory (3), antiviral (4) and anticancer activity (5–7). The potential use of aromatic/heterocyclic sulfonamides as carbonic anhydrase (CA) inhibitors has been little explored to date in the treatment of cancer. Acetazolamide (CAS 59-66-5) **I** was reported as a strong inhibitor of several carbonic anhydrase isozymes (8). It acts as a potential modulator of anticancer therapies in combination with different cytotoxic agents (alkylating agents, nucleoside analogs, etc.). Also, 5,7-diphenylpyrrolo[2,3-d]pyrimidines **II** were described as potent inhibitors of the tyrosine kinase C-Src, which is now considered as an attractive target of cancer therapy (9). In addition, it was reported that pyrrolopyrimidine U101033 (phase III clinical trials for ischemic brain injuries; have not yet CAS number) [(9-(2-morpholinyl)indole monohydrochloride hydrate] efficiently protected against hydroxy radical-induced lipid peroxidation that occurs deeply within the membrane bilayer (10). Due to our interest in the

development of novel anticancer agents, in this study, we report the synthesis of some novel pyrrole and pyrrolopyrimidine derivatives containing a biologically active sulfonamide moiety as analogs to **I** and **II**, respectively, hoping that these new compounds might show significant anticancer activity.



EXPERIMENTAL

Chemistry

Melting points were determined in open capillaries on a Gallenkamp melting point apparatus (Sanyo Gallenkamp, Southborough, UK) and are uncorrected. Pre-coated silica gel plates (silica gel, 60 F₂₅₄; Merck, Germany) were used for thin layer chromatography, dichloromethane/methanol (9.5 : 0.5, v/v) mixture was used as a developing solvent system and the spots were visualized by ultraviolet light and/or iodine. Infra-red spectra were recorded

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in KBr discs using IR-470 Shimadzu spectrometer (Shimadzu, Tokyo, Japan). NMR spectra in DMSO- d_6 were recorded on Bruker Ac-500 ultra shield NMR spectrometer (Bruker, Flawil, Switzerland) at 500 MHz, using TMS as internal standard and peak multiplicities are designed as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Elemental analyses were performed on Carlo Erba 1108 Elemental Analyzer (Heraeus, Hanau, Germany). All compounds were within $\pm 0.4\%$ of the theoretical values.

4-[2-(4-Bromophenyl)-2-oxoethylamino]benzenesulfonamide (3)

A mixture of sulfanilamide **1** (1.72 g, 0.01 mol) and 4-bromophenacylbromide **2** (2.77 g, 0.01 mol) was refluxed in N,N' -dimethylformamide (20 mL) in the presence of catalytic amount of triethylamine for 6 h. The solid obtained was filtered and recrystallized from ethanol to give **3**. Yield 89%, m.p. 232.6°C, IR: (ν_{\max} , cm^{-1}): 3358, 3255 (NH, NH_2), 3100 (CH arom.), 2970, 2863 (CH aliph.), 1685 (C=O), 1381, 1157 (SO_2). $^1\text{H-NMR}$ (DMSO- d_6 , D_2O , δ , ppm): 4.7 (s, 2H, CH_2), 6.6 (s, 1H, NH, D_2O exchangeable), 6.7, 7.5 (2d, 4H, Ar-H, AB system, $J = 7.1$ Hz), 7.8, 8.0 (2d, 4H, Ar-H, AB system, $J = 6.9$ Hz). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ , ppm): 49.4, 111.4 (2), 127.1, 127.7, 129.9 (2), 130.7 (2), 131.8 (2), 133.9, 150.8, 195.3. Analysis: calcd. for $\text{C}_{14}\text{H}_{13}\text{BrN}_2\text{O}_3\text{S}$ (369.23): C, 45.54; H, 3.55; N, 7.59%; found: C, 45.54; H, 3.31; N, 7.24%.

4-[2-Amino-4-(4-bromophenyl)-3-cyano-1H-pyrrol-1-yl]benzenesulfonamide (5)

A mixture of compound **3** (3.69 g, 0.01 mol) and malononitrile (0.66 g, 0.01 mol) in ethanol (20 mL) containing sodium ethoxide (0.5 g) was refluxed for 8 h. The reaction mixture was cooled and acidified with dil. HCl. The solid obtained was filtered and recrystallized from dioxane to give **5**. Yield 78%, m.p. 221.2°C, IR: (ν_{\max} , cm^{-1}): 3419, 3344, 3238 (NH_2), 3095 (CH arom.), 2187 (C=N), 1635 (C=N), 1342, 1176 (SO_2). $^1\text{H-NMR}$ (DMSO- d_6 , D_2O , δ , ppm): 6.1 (s, 2H, NH_2 , D_2O exchangeable), 7.0 (s, 1H, CH pyrrole), 7.5–7.9 (m, 10H, Ar-H + SO_2NH_2). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ , ppm): 70.5, 113.5, 117.5, 119.5, 121.3 (2), 125.2, 127.3, 131.2 (2), 131.6 (2), 132.3 (2), 139.5, 142.9, 148.5. Analysis: calcd. for $\text{C}_{17}\text{H}_{13}\text{BrN}_4\text{O}_2\text{S}$ (417.28): C, 48.93; H, 3.14; N, 13.43%; found: C, 48.71; H, 3.50; N, 13.16%.

4-[4-Amino-5-(4-bromophenyl)-7H-pyrrolo[2,3-d]pyrimidin-7-yl]benzenesulfonamide (6)

A solution of compound **5** (4.17 g, 0.01 mol) in formamide (20 mL) was refluxed for 8 h. The reaction mixture was cooled and then poured onto ice/cold water. The formed residue was recrystallized from dioxane to give **6**. Yield 75%, m.p. > 350°C, IR: (ν_{\max} , cm^{-1}): 3470, 3380, 3217 (NH_2), 3100 (CH arom.), 1620 (C=N), 1381, 1159 (SO_2). $^1\text{H-NMR}$ (DMSO- d_6 , D_2O , δ , ppm): 6.5 (s, 2H, NH_2 , D_2O exchangeable), 7.2 (s, 1H, CH pyrrole), 7.4–8.0 (m, 10H, Ar-H + SO_2NH_2), 8.6 (s, 1H, CH pyrimidine). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ , ppm): 100.9, 116.8, 120.4, 122.5, 123.2, 124.3, 126.7, 127.0, 128.5, 130.5, 131.7, 132.9, 139.9, 141.3, 152.6, 157.6, 160.2, 162.9. Analysis: calcd. for $\text{C}_{18}\text{H}_{14}\text{BrN}_5\text{O}_2\text{S}$ (444.31): C, 48.66; H, 3.18; N, 15.76%; found: C, 48.41; H, 3.46; N, 15.98%.

General procedure for synthesis of compounds 7a–m

A mixture of **5** (4.17 g, 0.01 mol) and aromatic aldehydes (0.01 mol.) in glacial acetic acid (20 mL) was refluxed for 4 h. The reaction mixture was cooled, filtered and the obtained solid was recrystallized from dioxane to give **7a–m**, respectively.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-methylbenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7a)

Yield 68%, m.p. 277.3°C, IR: (ν_{\max} , cm^{-1}): 3363, 3269 (NH_2), 3100 (CH arom.), 2970, 2816 (CH aliph.), 2202 (C=N), 1600 (C=N), 1328, 1166 (SO_2). $^1\text{H-NMR}$ (DMSO- d_6 , D_2O , δ , ppm): 2.4 (s, 3H, CH_3), 7.3 (s, 1H, CH pyrrole), 7.4–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.0 (s, 1H, N=CH). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ , ppm): 21.2, 78.7, 116.8, 119.4, 120.6, 124.0, 125.6, 126.5, 127.9, 129.2 (2), 129.6 (2), 129.7 (2), 131.3, 131.8, 132.4, 133.9 (2), 139.4, 143.0, 145.2, 147.0, 163.4. Analysis: calcd. for $\text{C}_{25}\text{H}_{19}\text{BrN}_4\text{O}_2\text{S}$ (518.04): C, 57.81; H, 3.69; N, 10.79%; found: C, 57.50; H, 3.88; N, 10.49%.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-hydroxybenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7b)

Yield 82%, m.p. 194.3°C, IR: (ν_{\max} , cm^{-1}): 3410 (OH), 3356, 3270 (NH_2), 3095 (CH arom.), 2966, 2846 (CH aliph.), 2208 (C=N), 1595 (C=N), 1346, 1161 (SO_2). $^1\text{H-NMR}$ (DMSO- d_6 , D_2O , δ , ppm): 6.9 (s, 1H, CH pyrrole), 7.0–8.0 (m, 14H, Ar-H + SO_2NH_2), 8.9 (s, 1H, N=CH), 10.5 (s, 1H, OH, D_2O exchangeable). $^{13}\text{C-NMR}$ (DMSO- d_6 , δ , ppm): 78.2, 117.0, 117.6, 118.8, 119.5, 120.5, 121.3 (2), 125.5, 126.5, 127.3, 128.3 (2), 131.4 (2), 131.8 (2), 132.3 (2), 139.5, 142.8, 148.5, 162.1, 163.1. Analysis:

calcd. for $C_{24}H_{17}BrN_4O_3S$ (521.38): C, 55.29; H, 3.29; N, 10.75%. found: C, 55.50; H, 3.10; N, 10.66%.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-fluorobenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7c)

Yield 80%, m.p. 142.9°C, IR: (ν_{max} , cm^{-1}) 3300, 3270 (NH_2), 3055 (CH arom.), 2971, 2836 (CH aliph.), 2210 (C=N), 1598 (C=N), 1372, 1165 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.3(s, 1H, CH pyrrole), 7.4–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.1 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 78.9, 116.4 (2), 116.7, 119.7, 124.1, 125.2 (2), 126.5, 127.1, 127.3 (2), 127.8, 128.0 (2), 131.9 (2), 132.3 (2), 139.5, 143.1, 146.6, 162.2, 165.8. Analysis: calcd. for $C_{24}H_{16}BrFN_4O_2S$ (522.01): C, 55.08; H, 3.08; N, 10.70%; found: C, 55.25; H, 3.31; N, 10.59%.

4-[4-(4-Bromophenyl)-3-cyano-2-(2-methoxybenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7d)

Yield 56%, m.p. 137.6°C, IR: (ν_{max} , cm^{-1}): 3280, 3263 (NH_2), 3074 (CH arom.), 2941, 2839 (CH aliph.), 2212 (C=N), 1597 (C=N), 1340, 1163 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 3.9 (s, 3H, OCH_3), 7.0 (s, 1H, CH pyrrole), 7.2–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.2 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 56.0, 110.2, 112.6, 116.8 (2), 119.8, 120.9, 122.7, 123.0 (2), 124.4, 128.0, 129.6 (2), 131.8 (2), 132.3, 134.7, 136.6, 136.7, 139.4, 143.0, 147.3, 160.0, 161.4. Analysis: calcd. for $C_{25}H_{19}BrN_4O_3S$ (535.41): C, 56.08; H, 3.58; N, 10.46%; found: C, 56.26; H, 3.31; N, 10.71%.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-methoxybenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7e)

Yield 66%, m.p. 141.2°C, IR: (ν_{max} , cm^{-1}): 3338, 3259 (NH_2), 3100 (CH arom.), 2970, 2860 (CH aliph.), 2212 (C=N), 1593 (C=N), 1370, 1166 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 3.8 (s, 3H, OCH_3), 7.1 (s, 1H, CH pyrrole), 7.2–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.0 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 56.0, 78.3, 114.6 (2), 116.9, 119.1, 120.5, 123.9 (2), 125.6, 126.5, 127.7, 127.9 (2), 129.6 (2), 131.2 (2), 131.8 (2), 139.5, 142.9, 147.5, 162.9, 164.1. Analysis: calcd. for $C_{25}H_{19}BrN_4O_3S$ (535.41): C, 56.08; H, 3.58; N, 10.46%; found: C, 56.30; H, 3.36; N, 10.68%.

4-[2-(Benzo[d][1,3]dioxol-5-ylmethyleneamino)-4-(4-bromophenyl)-3-cyano-1H-pyrrol-1-yl]benzenesulfonamide (7f)

Yield 66%, m.p. 111.9°C, IR: (ν_{max} , cm^{-1}): 3346, 3261 (NH_2), 3100 (CH arom.), 2920, 2860 (CH aliph.), 2210 (C=N), 1587 (C=N), 1340, 1165 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 6.1 (s, 2H, CH_2), 7.0 (s, 1H, CH pyrrole), 7.1–7.9 (m, 13H, Ar-H + SO_2NH_2), 8.9 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 102.2, 106.2, 108.6, 116.8, 119.2, 120.5, 123.9, 124.4 (2), 124.8, 126.5, 127.9, 128.5, 129.6 (2), 131.4 (2), 131.8 (2), 139.4, 143.0, 148.3, 151.6, 152.7, 162.5. Analysis: calcd. for $C_{25}H_{17}BrN_4O_4S$ (549.39): C, 54.65; H, 3.12; N, 10.20%; found: C, 54.91; H, 3.39; N, 10.46%.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-(dimethylamino)benzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7g)

Yield 76%, m.p. 99.7°C, IR: (ν_{max} , cm^{-1}): 3300, 3246 (NH_2), 3100 (CH arom.), 2920, 2870 (CH aliph.), 2206 (C=N), 1593 (C=N), 1373, 1165 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 3.0 (s, 6H, $2CH_3$), 6.7 (s, 1H, CH pyrrole), 7.0–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.6 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 41.1 (2), 111.5, 115.1 (2), 117.3, 119.4, 120.4, 124.4 (2), 125.5, 126.6, 127.3, 127.9 (2), 131.5 (2), 132.3 (2), 134.3 (2), 139.7, 142.1, 148.7, 153.3, 162.7. Analysis: calcd. for $C_{26}H_{22}BrN_5O_2S$ (548.45): C, 56.94; H, 4.04; N, 12.77%; found: C, 56.68; H, 4.36; N, 12.55%.

4-[4-(4-Bromophenyl)-3-cyano-2-(4-nitrobenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7h)

Yield 85%, m.p. 318.5°C, IR: (ν_{max} , cm^{-1}): 3373, 3269 (NH_2), 3100 (CH arom.), 2910, 2860 (CH aliph.), 2214 (C=N), 1593 (C=N), 1398, 1165 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.5 (s, 1H, CH pyrrole), 7.7–8.0 (m, 14H, Ar-H + SO_2NH_2), 9.2 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 79.7, 116.4, 120.8 (2), 124.2 (2), 124.7, 125.8 (2), 126.6, 128.0 (2), 130.6 (2), 131.0 (2), 131.9 (2), 139.2, 140.4, 143.3, 145.3, 149.4, 160.6. Analysis: calcd. for $C_{24}H_{16}BrN_5O_2S$ (549.01): C, 52.37; H, 2.93; N, 12.72%; found: C, 52.10; H, 2.66; N, 12.44%.

4-[4-(4-Bromophenyl)-3-cyano-2-(2,4-dinitrobenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7i)

Yield 77%, m.p. 210.2°C, IR: (ν_{max} , cm^{-1}): 3300, 3211 (NH_2), 3095 (CH arom.), 2930, 2860 (CH aliph.), 2218 (C=N), 1583 (C=N), 1346, 1165 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 6.9 (s, 1H, CH pyrrole), 7.1–7.9 (m, 13H, Ar-H + SO_2NH_2), 10.3 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 104.4,

119.7, 120.6, 123.6, 126.6, 127.6 (2), 128.5, 129.0, 129.5 (2), 131.5 (2), 131.9, 132.2, 132.4 (2), 134.4, 134.8, 146.0, 148.9, 149.5, 151.5, 156.0. Analysis: calcd. for $C_{24}H_{15}BrN_6O_6S$ (595.38): C, 48.42; H, 2.54; N, 14.12%; found: C, 48.76; H, 2.26; N, 14.51%.

4-[4-(4-Bromophenyl)-2-(4-chlorobenzylideneamino)-3-cyano-1H-pyrrol-1-yl]benzenesulfonamide (7j)

Yield 79%, m.p. 164.6°C, IR: (ν_{max} , cm^{-1}): 3305, 3255 (NH_2), 3095 (CH arom.), 2910, 2861 (CH aliph.), 2212 (C=N), 1593 (C=N), 1338, 1165 (SO_2), 719 (C-Cl). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.0 (s, 1H, CH pyrrole), 7.4–7.9 (m, 14H, Ar-H + SO_2NH_2), 9.1 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 79.1, 116.6, 119.9, 120.7, 124.3 (2), 125.7, 126.6, 127.8 (2), 129.3 (2), 131.2 (2), 131.9 (2), 133.8, 134.7 (2), 137.5, 139.3, 143.1, 146.3, 162.0. Analysis: calcd. for $C_{24}H_{16}BrClN_4O_2S$ (537.98): C, 53.40; H, 2.99; N, 10.38%; found: C, 53.67; H, 2.68; N, 10.76%.

4-[4-(4-Bromophenyl)-3-cyano-2-(2,4-dichlorobenzylideneamino)-1H-pyrrol-1-yl]benzenesulfonamide (7k)

Yield 86%, m.p. 115.6°C, IR: (ν_{max} , cm^{-1}): 3346, 3265 (NH_2), 3088 (CH arom.), 2926, 2836 (CH aliph.), 2216 (C=N), 1581 (C=N), 1379, 1166 (SO_2), 719, 825 (2 C-Cl). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.1 (s, 1H, CH pyrrole), 7.2–7.9 (m, 13H, Ar-H + SO_2NH_2), 10.2 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 79.6, 120.6, 125.7, 125.9, 126.5 (2), 127.3, 128.2, 129.2, 130.6 (2), 131.0 (2), 132.4, 133.6, 136.3, 137.1 (2), 139.7 (2), 144.9, 145.3, 145.5, 156.8. Analysis: calcd. for $C_{24}H_{15}BrCl_2N_4O_2S$ (574.27): C, 50.19; H, 2.63; N, 9.76%; found: C, 50.47; H, 2.37; N, 9.50%.

4-[4-(4-Bromophenyl)-3-cyano-2-[(2-methoxynaphthalen-1-yl)methylene-amino]-1H-pyrrol-1-yl]benzenesulfonamide (7l)

Yield 65%, m.p. 278.2°C, IR: (ν_{max} , cm^{-1}): 3346, 3255 (NH_2), 3074 (CH arom.), 2971, 2842 (CH aliph.), 2210 (C=N), 1624 (C=N), 1371, 1151 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 4.0 (s, 3H, OCH_3), 7.4 (s, 1H, CH pyrrole), 7.5–8.0 (m, 16H, Ar-H + SO_2NH_2), 9.9 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 57.1, 104.3, 113.5, 114.5, 117.0, 117.6, 119.6, 120.0 (2), 120.5, 124.3, 125.1, 126.4, 126.5, 128.1 (2), 128.5, 128.7, 130.8 (2), 131.5, 131.8 (2), 136.2, 139.7, 143.5, 148.4, 160.1, 161.5. Analysis: calcd. for $C_{29}H_{21}BrN_4O_3S$ (584.05): C, 59.49; H, 3.62; N, 9.57%; found: C, 59.21; H, 3.9137; N, 9.81%.

4-[4-(4-Bromophenyl)-3-cyano-2-[(4-methoxynaphthalen-1-yl)methylene-amino]-1H-pyrrol-1-yl]benzenesulfonamide (7m)

Yield 73%, m.p. 181.2°C, IR: (ν_{max} , cm^{-1}): 3327, 3310 (NH_2), 3100 (CH arom.), 2955, 2846 (CH aliph.), 2208 (C=N), 1590 (C=N), 1370, 1157 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 4.0 (s, 3H, OCH_3), 7.1–8.0 (m, 16H, Ar-H + SO_2NH_2), 8.9 (s, 1H, CH pyrrole), 9.5 (s, 1H, N=CH). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 56.2, 104.0, 104.7, 117.2, 119.2, 120.5, 122.0, 122.3 (2), 122.5, 124.0, 124.2, 124.6, 124.8, 125.9, 126.1, 126.5, 127.9 (2), 131.5 (2), 134.5 (2), 139.6, 140.3, 143.2, 148.1, 159.1, 160.1. Analysis: calcd. for $C_{29}H_{21}BrN_4O_3S$ (585.47): C, 59.49; H, 3.62; N, 9.57%; found: C, 59.70; H, 3.4137; N, 9.19%.

4-[5-(4-Bromophenyl)-4-oxo-3,4-dihydropyrrolo[2,3-d]pyrimidin-7-yl]benzenesulfonamide (8)

A solution of compound **5** (4.17 g, 0.01 mol.) in formic acid (20 mL) was refluxed for 6 h. The reaction mixture was poured onto ice/water and the solid obtained was recrystallized from dioxane to give **8**. Yield 81%, m.p. 313.7°C, IR: (ν_{max} , cm^{-1}): 3320, 3236, 3156 (NH, NH_2), 3094 (CH arom.), 1683 (C=O), 1591 (C=N), 1386, 1161 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.5 (s, 1H, CH pyrrole), 7.6–8.0 (m, 10H, Ar-H + SO_2NH_2), 8.1 (s, 1H, CH pyrimidine), 12.3 (s, 1H, NH, D_2O exchangeable). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 106.3, 119.7, 120.3, 121.5 (2), 124.4, 126.7, 127.9 (2), 130.8 (2), 132.3 (2), 141.1, 142.2, 145.0, 148.2, 158.4. Analysis: calcd. for $C_{18}H_{13}BrN_4O_3S$ (445.29): C, 48.55; H, 2.94; N, 12.58%; found: C, 48.30; H, 2.65; N, 12.84%.

N-[4-(4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1H-pyrrol-2-yl]-2,2,2-trifluoroacetamide (9)

A solution of compound **5** (4.17 g, 0.01 mol.) in trifluoroacetic anhydride (15 mL) was refluxed for 20 h. The solid obtained was recrystallized from ethanol to give **9**. Yield 69%, m.p. 206.5°C, IR: (ν_{max} , cm^{-1}): 3309, 3291 (NH_2), 3100 (CH arom.), 2202 (C=N), 1681 (C=O), 1610 (C=N), 1336, 1151 (SO_2). 1H -NMR (DMSO- d_6 , D_2O , δ , ppm): 7.1 (s, 1H, CH pyrrole), 7.2–8.4 (m, 10H, Ar-H + SO_2NH_2), 13.8 (s, 1H, NH, D_2O exchangeable). ^{13}C -NMR (DMSO- d_6 , δ , ppm): 118.6, 119.4, 120.2, 123.8, 124.1, 124.5, 124.9, 126.5, 126.8, 127.1 (2), 130.3, 130.5, 131.0, 131.5, 131.7, 138.9, 147.7, 159.9. Analysis: calcd. for $C_{19}H_{12}BrF_3N_4O_3S$ (513.29): C, 44.46; H, 2.36; N, 10.92%; found: C, 44.12; H, 2.71; N, 10.66%.

***N*-[4-(4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl]acetamide (10)**

A solution of compound **5** (4.17 g, 0.01 mol.) in acetic anhydride (20 mL) was refluxed for 5 min. The reaction mixture was concentrated and the solid separated was recrystallized from ethanol to give **10**. Yield 81%, m.p. 113.0°C, IR: (ν_{\max} , cm^{-1}): 3389, 3344, 3238 (NH, NH₂), 3095 (CH arom.), 2981, 2862 (CH aliph.), 2189 (C=N), 1685 (C=O), 1635 (C=N), 1340, 1163 (SO₂). ¹H-NMR (DMSO-d₆, D₂O, δ , ppm): 2.0 (s, 3H, COCH₃), 6.1 (s, 1H, CH pyrrole), 7.0–8.0 (m, 10H, Ar-H + SO₂NH₂), 10.2 (s, 1H, NH, D₂O exchangeable). ¹³C-NMR (DMSO-d₆, δ , ppm): 22.3, 113.5, 117.5, 119.5, 121.3, 123.0, 124.8, 125.8, 127.8, 131.3 (2), 132.3 (2), 134.3 (2), 139.2, 142.9, 148.5, 170.0. Analysis: calcd. for C₁₉H₁₅BrN₄O₃S (459.32): C, 49.68; H, 3.29; N, 12.20%; found: C, 49.36; H, 3.51; N, 12.56%.

***N*-acetyl-*N*-[4-(4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl]acetamide (11)**

A solution of compound **5** (4.17 g, 0.01 mol.) in acetic anhydride (20 mL) was refluxed for 24 h. The reaction mixture was concentrated and the solid obtained was recrystallized from dioxane to give **11**. Yield 59%, m.p. 142.8°C, IR: (ν_{\max} , cm^{-1}): 3495, 3122 (NH₂), 3055 (CH arom.), 2920, 2862 (CH aliph.), 2227 (C=N), 1734, 1718 (2 C=O), 1593 (C=N), 1369, 1161 (SO₂). ¹H-NMR (DMSO-d₆, D₂O, δ , ppm): 2.4 (s, 6H, 2COCH₃), 7.4 (s, 1H, CH pyrrole), 7.5–8.1 (m, 10H, Ar-H + SO₂NH₂). ¹³C-NMR (DMSO-d₆, δ , ppm): 21.0 (2), 113.9, 120.4, 120.8, 120.9, 121.8 (2), 124.9, 125.3, 128.8 (2), 129.5 (2), 133.5 (2), 139.5, 140.1, 146.5, 171.8 (2). Analysis: calcd. for C₂₁H₁₇BrN₄O₄S (501.35): C, 50.31; H, 3.42; N, 11.18%; found: C, 50.63; H, 3.12; N, 11.41%.

***N*-[4-(4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl]-2-chloroacetamide (13)**

A solution of compound **5** (4.17 g, 0.01 mol.) in chloroacetylchloride (20 mL) was refluxed for 1 h. The reaction mixture was poured onto ice/water and the solid obtained was recrystallized from dioxane to give **13**. Yield 80%, m.p. 154.4°C, IR: (ν_{\max} , cm^{-1}): 3470, 3380, 3210 (NH, NH₂), 3100 (CH arom.), 2956, 2817 (CH aliph.), 2223 (C=N), 1707 (C=O), 1627 (C=N), 1388, 1149 (SO₂), 725 (C-Cl). ¹H-NMR (DMSO-d₆, D₂O, δ , ppm): 4.2 (s, 2H, CH₂), 7.4 (s, 1H, CH pyrrole), 7.5–7.9 (m, 10H, Ar-H + SO₂NH₂), 8.2 (s, 1H, NH, D₂O exchangeable). ¹³C-NMR (DMSO-d₆, δ , ppm): 42.4, 89.5, 114.7, 119.4, 121.3, 124.9 (2), 125.1, 128.5, 129.3, 130.5, 131.1, 131.4, 132.3, 133.1, 138.8, 142.6, 149.7, 166.8.

Analysis: calcd. for C₁₉H₁₄BrClN₄O₃S (493.76): C, 46.22; H, 2.86; N, 11.35%; found: C, 46.52; H, 2.67; N, 11.14%.

***N*-[4-(4-bromophenyl)-3-cyano-1-(4-sulfamoylphenyl)-1*H*-pyrrol-2-yl]-2-chloro-*N*-(2-chloroacetyl)acetamide (14)**

A solution of compound **5** (4.17 g, 0.01 mol) in chloroacetylchloride (20 mL) was refluxed for 16 h. The solid obtained was recrystallized from acetic acid to give **14**. Yield 64%, m.p. 359.2°C, IR: (ν_{\max} , cm^{-1}): 3253, 3190 (NH₂), 3088 (CH arom.), 2944, 2865 (CH aliph.), 2223 (C=N), 1720, 1705 (2 C=O), 1591 (C=N), 1388, 1174 (SO₂), 727 (C-Cl). ¹H-NMR (DMSO-d₆, D₂O, δ , ppm): 4.2 (s, 4H, 2 CH₂), 7.3 (s, 1H, CH pyrrole), 7.4–8.0 (m, 10H, Ar-H + SO₂NH₂). ¹³C-NMR (DMSO-d₆, δ , ppm): 42.1, 42.8, 89.7, 114.6, 119.4, 120.6, 123.3 (2), 124.8, 125.1, 125.8, 127.8 (2), 129.1 (2), 131.0, 131.9, 133.0, 138.2, 140.5, 165.2, 166.8. Analysis: calcd. for C₂₁H₁₅BrCl₂N₄O₄S (570.24): C, 44.23; H, 2.65; N, 9.83%; found: C, 44.51; H, 2.36; N, 10.19%.

Molecular docking

All the molecular modeling studies were carried out on an Intel Pentium 1.6 GHz processor, 512 MB memory with Windows XP operating system using Molecular Operating Environment (MOE, 10.2008) software. All the minimizations were performed with MOE until a RMSD gradient of 0.05 kcal mol⁻¹Å⁻¹ with MMFF94X force field and the partial charges were automatically calculated. The X-ray crystallographic structure of c-Src complex with its ligand (PDB ID: 1YOL) was obtained from the protein data bank. The enzyme was prepared for docking studies where: (i) Ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. (iv) The obtained model was then used in predicting the ligand–enzymes interactions at the active site (Table 1).

***In vitro* antitumor activity**

Human tumor breast cell line (MCF7) was used in this study. The cytotoxic activity was measured *in vitro* for the newly synthesized compounds using the sulfo-rhodamine-B stain (SRB) assay using the method of Skehan et al. (11). The *in vitro* anticancer screening was done by the pharmacology unit at the National Cancer Institute, Cairo University. Cells were plated in 96-multiwell plate

(10⁴ cells/well) for 24 h before treatment with the compound(s) to allow attachment of cell to the wall of the plate. The tested compounds were dissolved in dimethyl sulfoxide. Different concentrations of the compound under test (10, 25, 50, and 100 μ M) were added to the cell monolayer. Triplicate wells were prepared for each individual concentration. Monolayer cells were incubated with the compound(s) for 48 h at 37°C and in atmosphere of 5% CO₂. After 48 h, cells were fixed, washed and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. An 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 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. The molar concentration required for 50% inhibition of cell viability (IC₅₀) was calculated and compared to the reference drug doxorubicin (CAS, 25316-40-9). The surviving fractions were expressed as the means \pm standard error and the results are given in Table 2.

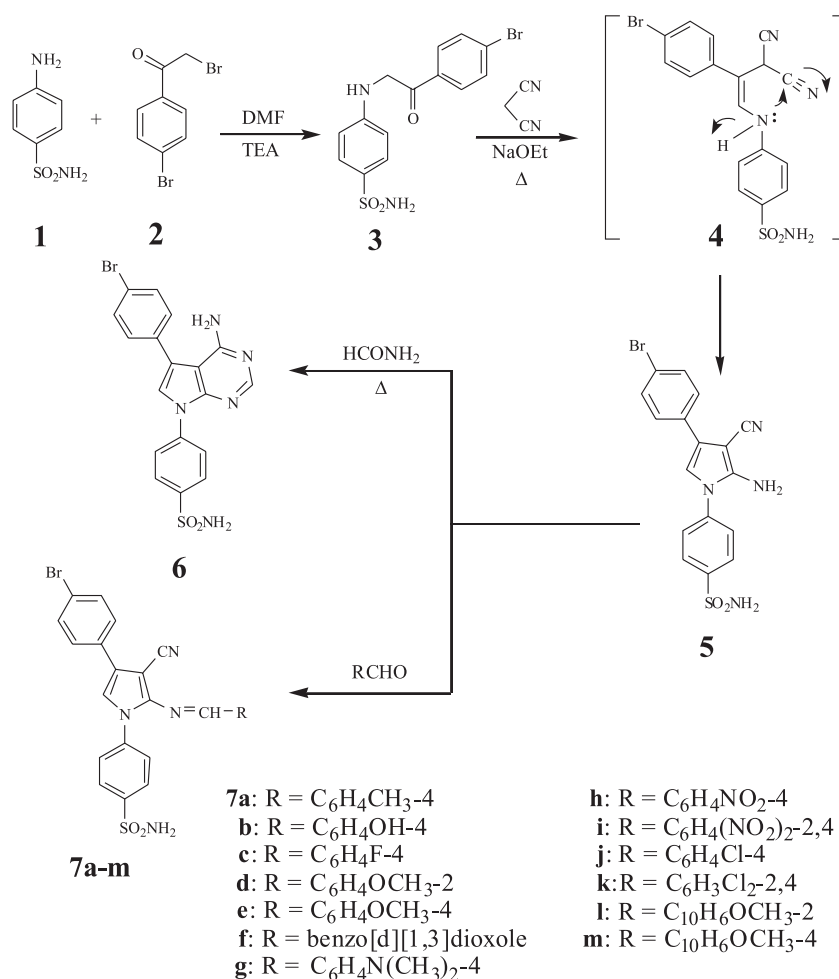
RESULTS AND DISCUSSION

Chemistry

The synthesized compounds were designed with the aim of exploring their anticancer activity. In this investigation, a novel series of bromopyrroles **5**, **7a–m**, **9–11**, **13**, **14** and bromopyrrolopyrimidines **6**, **8** having a biologically active sulfonamide moieties were synthesized to evaluate their *in vitro* anticancer activity. Thus, interaction of sulfanilamide **1** with 4-

Table 1. Binding scores and amino acid interactions of the docked compounds on the active site of c-Src.

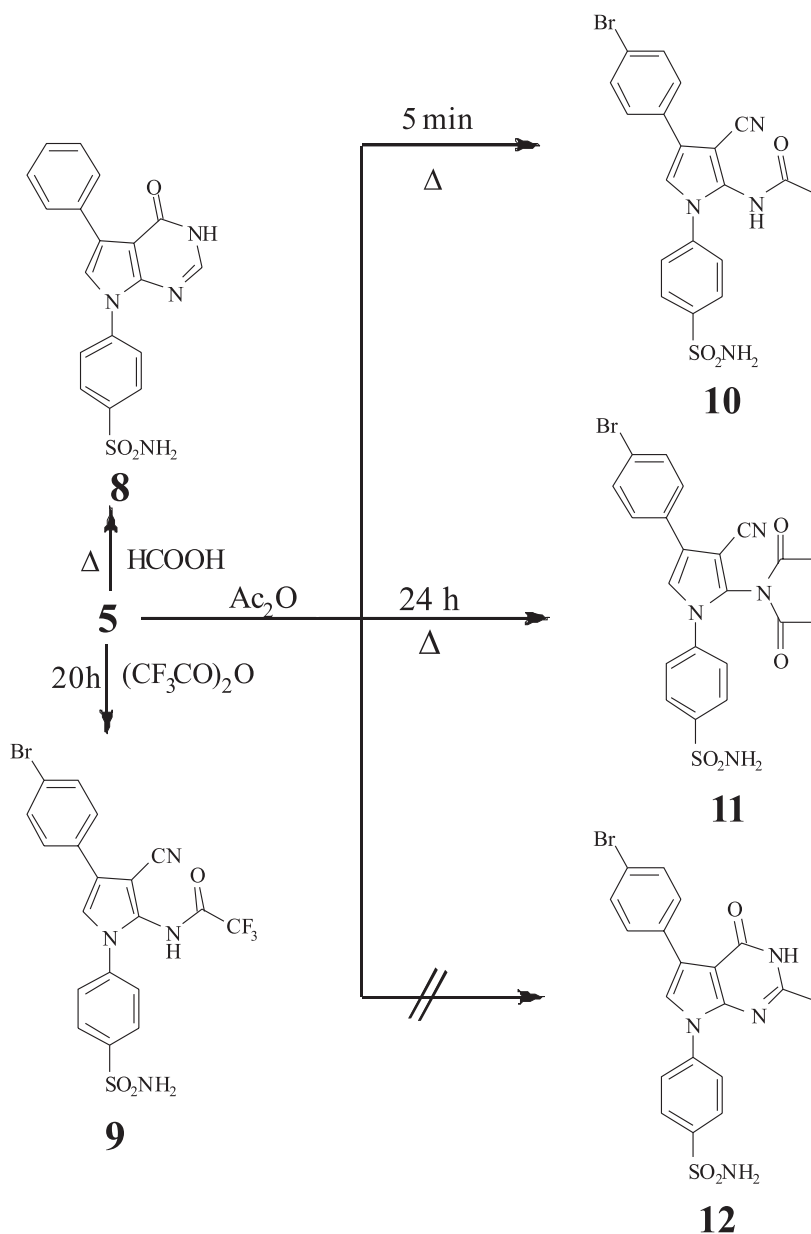
Compd. No.	S Kcal/mol	Amino acid interactions	Interacting groups	H bond length Å
5	-13.6201	Asp 350, Met 343 Ser 347	NH ₂ N pyrimidine	3.22, 2.22 3.09
6	-16.1961	Glu 341, Met 343 Asp 350, Ser 347	NH ₂ , N pyrimidine SO ₂ NH ₂ , SO ₂ NH ₂	1.94, 2.89 1.42, 3.02
7a	-17.7147	Glu 312, Asp 436 Asp 350	SO ₂ NH ₂ , SO ₂ NH ₂ CN	2.43, 2.01 3.74
7b	-9.3848	Met 343, Asp 350 Ser 347	OH, SO ₂ NH ₂ SO ₂	2.89, 1.98 3.47
7c	-10.4441	Asp 350	SO ₂	3.04
7d	-14.8247	Glu 281, Asp 350 Asp 350	OCH ₃ , SO ₂ NH ₂	3.49, 1.67 3.32
7e	-11.6741	Asp 350	SO ₂ NH ₂	2.34–1.80
7f	-12.3196	Asp 350	SO ₂ NH ₂	3.01
7g	-20.4112	Asp 350	N=C	3.11
7h	-17.8049	Glu 312, Asp 406	SO ₂ NH ₂ , SO ₂ NH ₂	2.14, 2.15
7i	-15.5585	Asp 350	CN	3.74
7j	-13.7170	Met 343	SO ₂ , SO ₂ NH ₂	3.25, 2.01
7k	-19.0188	Asp 350	SO ₂ NH ₂	2.40
7l	-22.8663	Gly 281	SO ₂ NH ₂	3.28, 3.15
7m	-10.4936	Asp 350	SO ₂ NH ₂	1.78
8	-3.1180	Asp 350, Ser 347	SO ₂ NH ₂ , SO ₂ NH ₂	1.40, 1.97
9	-11.6900	Asp 350, Asp 406 Glu 312	SO ₂ NH ₂ , SO ₂ NH ₂ SO ₂ NH ₂	3.48, 3.05 2.16
10	-16.0731	Asp 350	SO ₂ NH ₂	1.52
11	-10.0602	Asp 350	SO ₂ NH ₂	2.51
13	-13.0866	Ala 382	C=O	2.43
14	-12.9995	Asp 350	SO ₂ NH ₂	1.57

Scheme 1. Formation of pyrrolo and pyrrolopyrimidine derivatives **5-7a-m**

bromophenacyl bromide **2** in DMF containing a catalytic amount of triethylamine gave 4-(2-(4-bromophenyl)-2-oxoethyl-amino)benzenesulfonamide **3**, which upon interaction with malononitrile in refluxing ethanol containing sodium ethoxide furnished the strategic starting material, pyrrole-2-amino-3-carbonitrile **5** (Scheme 1). The formation of compound **5** proceeded *via* initial formation of the intermediate **4** followed by intramolecular cyclization to give **5**. The structure of compound **3** was verified by elemental analysis and spectral data. The IR spectrum of compound **3** showed the presence of the characteristic bands for (NH, NH₂), (C=O) and (SO₂). Also, ¹H-NMR spectrum indicated the presence of a singlet at 4.7 ppm, which could be assigned to CH₂ group. The IR spectrum of compound **5** exhibited bands for (NH₂), (C=N) and (SO₂)

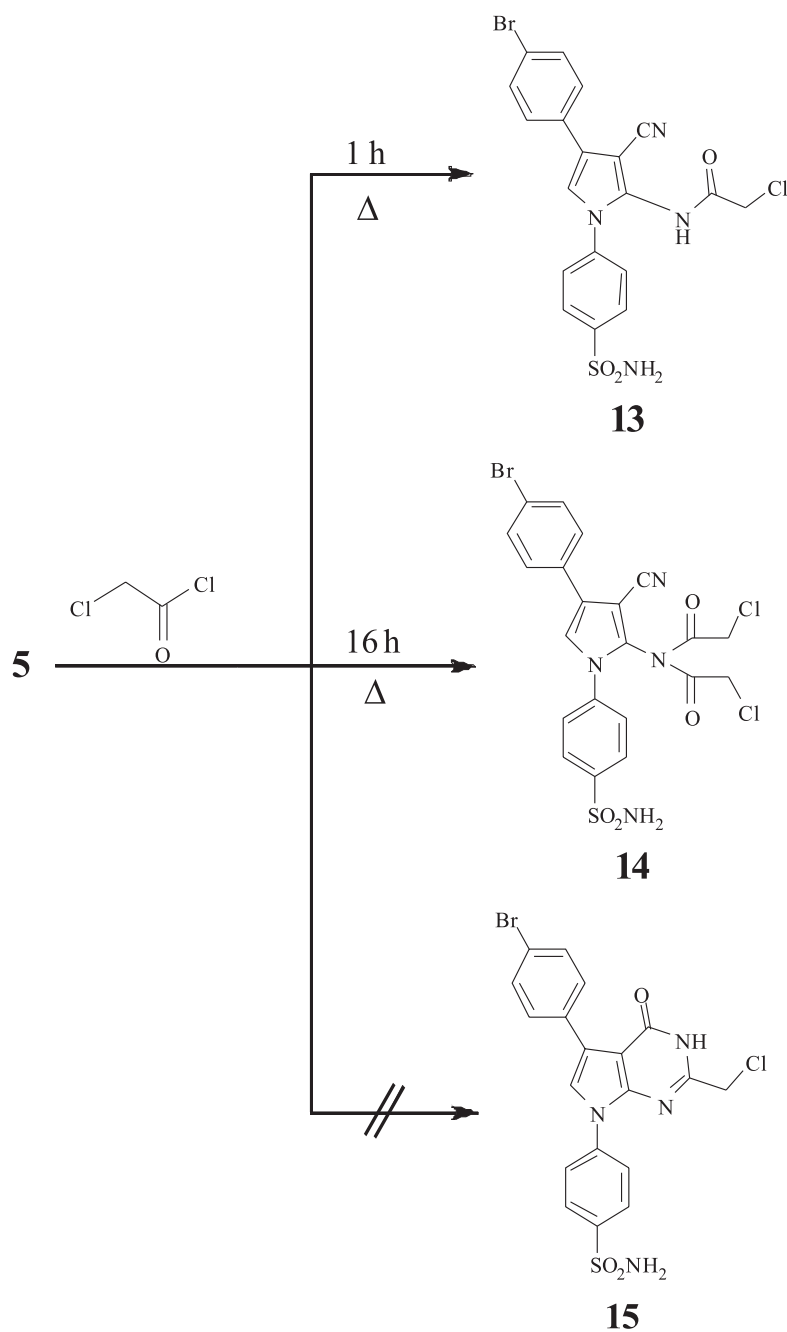
groups. In addition, ¹H-NMR spectrum of compound **5** revealed signals at 6.1 ppm due to NH₂ group and 7.9 ppm for SO₂NH₂ group.

Interaction of compound **5** with formamide caused cyclization to give pyrrolopyrimidine derivative **6** (Scheme 1). Its IR spectrum showed the absence of (C=N) band, which confirms the cyclization and formation of pyrrolopyrimidine system **6**. In addition, the behavior of compound **5** towards carbonyl compounds was studied. Thus, the reaction of compound **5** with aromatic aldehydes in acetic acid yielded Schiff's bases **7a-m**. The structures of compounds **7a-m** were confirmed by elemental analyses, IR, ¹H-NMR and ¹³C-NMR spectra. The IR spectra of compounds **7a-m** revealed the presence of (C=N) band. Also, ¹H-NMR spectra showed the presence of singlet for (N=CH) group.

Scheme 2. Formation of new pyrrolo and pyrrolopyrimidine derivatives **8–11**

Refluxing compound **5** in formic acid caused cyclization *via* elimination of water to give the pyrrolopyrimidine derivative **8** (Scheme 2). Its IR spectrum exhibited the absence of (C=N) band, which confirms the cyclization and formation of pyrrolopyrimidine system **8**. The reactivity of compound **5** towards acid anhydride in different time of reflux was observed. Thus, reaction of compound **5** with acetic anhydride for 5 min furnished the monoacetyl derivative **10**. On the other hand, when

compound **5** was reacted with acetic anhydride for long time (24 h), the corresponding diacetyl derivative **11** was obtained instead of the cyclic system pyrrolopyrimidine derivative **12** on the basis of elemental analysis and IR spectrum, which showed the presence of (C=N) band. When compound **5** was reacted with trifluoroacetic anhydride for long time (20 h), the corresponding monoacetyl derivative **9** was obtained, on the basis of elemental analysis and IR spectrum, which showed the presence of (C=N)

Scheme 3. Formation of some novel pyrrole derivatives **13** and **14**

band. $^1\text{H-NMR}$ spectrum of **11** showed singlet at 2.4 ppm for two acetyl groups.

On the other hand, when compound **5** was reacted with chloroacetylchloride for short time (1 h), the corresponding monoacetylchloride derivative **13** was obtained while, applying the same reaction

for long time (16 h), the corresponding diacetylchloride derivative **14** was obtained in a good yield rather than the cyclic pyrrolopyrimidine derivative **15**, based on the elemental analyses and spectral data. The IR spectrum of compound **13** showed the presence of (C=N), (C=O) and (C-Cl) bands. $^1\text{H-}$

Table 2. *In vitro* anticancer screening of the synthesized compounds against human breast cancer cell line (MCF7).

Compound	Compound concentration (μM)				IC_{50} (μM)
	10 μM	25 μM	50 μM	100 μM	
	Surviving fraction (mean \pm SE)*				
Doxorubicin	0.314 \pm 0.032	0.309 \pm 0.016	0.251 \pm 0.023	0.266 \pm 0.032	8.02
5	0.327 \pm 0.121	0.273 \pm 0.043	0.233 \pm 0.011	0.255 \pm 0.020	7.56
6	0.340 \pm 0.090	0.294 \pm 0.021	0.246 \pm 0.110	0.256 \pm 0.002	7.56
7a	0.275 \pm 0.113	0.265 \pm 0.005	0.323 \pm 0.010	0.349 \pm 0.032	6.74
7b	0.301 \pm 0.121	0.244 \pm 0.055	0.236 \pm 0.001	0.238 \pm 0.012	7.01
7c	0.300 \pm 0.090	0.247 \pm 0.035	0.238 \pm 0.023	0.311 \pm 0.066	7.34
7d	0.345 \pm 0.133	0.262 \pm 0.100	0.313 \pm 0.043	0.319 \pm 0.065	7.84
7e	0.234 \pm 0.033	0.225 \pm 0.111	0.248 \pm 0.015	0.279 \pm 0.076	6.47
7f	0.256 \pm 0.067	0.362 \pm 0.021	0.345 \pm 0.034	0.235 \pm 0.041	7.90
7g	0.252 \pm 0.084	0.265 \pm 0.142	0.231 \pm 0.101	0.264 \pm 0.102	6.46
7h	0.338 \pm 0.066	0.261 \pm 0.031	0.234 \pm 0.116	0.263 \pm 0.131	7.56
7i	0.235 \pm 0.019	0.228 \pm 0.008	0.261 \pm 0.032	0.256 \pm 0.056	6.46
7j	0.273 \pm 0.191	0.205 \pm 0.018	0.237 \pm 0.011	0.251 \pm 0.007	6.74
7k	0.331 \pm 0.012	0.310 \pm 0.110	0.295 \pm 0.044	0.299 \pm 0.033	8.15
7l	0.334 \pm 0.061	0.319 \pm 0.029	0.287 \pm 0.017	0.289 \pm 0.049	6.70
7m	0.391 \pm 0.028	0.300 \pm 0.015	0.299 \pm 0.006	0.311 \pm 0.112	8.45
8	0.273 \pm 0.004	0.315 \pm 0.017	0.276 \pm 0.003	0.287 \pm 0.001	6.74
9	0.361 \pm 0.011	0.379 \pm 0.089	0.298 \pm 0.042	0.321 \pm 0.058	7.84
10	0.353 \pm 0.071	0.323 \pm 0.006	0.273 \pm 0.027	0.222 \pm 0.010	7.56
11	0.288 \pm 0.055	0.229 \pm 0.091	0.240 \pm 0.042	0.242 \pm 0.080	6.74
13	0.324 \pm 0.010	0.340 \pm 0.021	0.294 \pm 0.069	0.316 \pm 0.049	7.29
14	0.332 \pm 0.023	0.273 \pm 0.011	0.285 \pm 0.048	0.234 \pm 0.052	7.29

* Each value is the mean of three values \pm standard error (SE)

NMR spectrum of **13** revealed the presence of a singlet at 4.2 ppm assigned for CH_2 group. The IR spectrum of **14** revealed the presence of ($\text{C}=\text{N}$) at 2223 cm^{-1} and $2\text{C}=\text{O}$ at 1720 and 1705 cm^{-1} . The ^1H -NMR spectrum of **14** exhibited singlet at 4.2 ppm due to two CH_2 groups (Scheme 3).

Molecular docking

Several classes of inhibitors are currently used to inhibit the activity of c-Src in a number of cell types. However, they often show poor selectivity within the c-Src family, which in mammals comprises at least eight members involved in many key functions of the cell (12). Recently, it has been shown that c-Src inhibitors of the pyrrolopyrimidine class exhibit a powerful inhibitory activity and a several-fold greater selectivity for c-Src against most tyrosine kinases (13–15), suggesting that c-Src

can be activated downstream of receptor activator of NF- κB (RANK) (16), a member of the tumor necrosis factor (TNF) receptor superfamily that is involved in cell differentiation, function, and survival (17–19).

In order to realize the aim of the present investigations, the authors have performed molecular docking of the synthesized compounds on the active sites of c-Src, which may provide an understanding of their effect as antitumor agents. The protein data bank file (PDB ID:1YOL) was selected for this purpose. The file contains c-Src enzyme co-crystallized with a pyrrolopyrimidine ligand. All docking procedures were achieved by MOE (Molecular Operating Environment) software 10.2008 provided by chemical computing group, Canada. Docking on the active site of c-Src enzyme was performed for all synthesized compounds. Docking protocol was verified by redocking of the co-crystal-

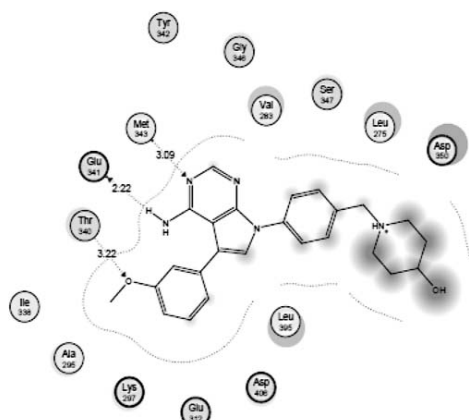


Figure 1. Pyrrolopyrimidine ligand on the active site of c-Src enzyme

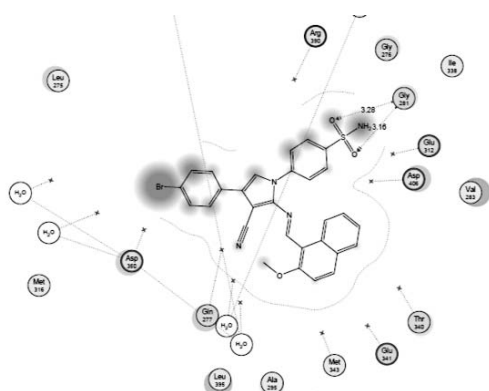


Figure 3. Compound **7l** on the active site of c-Src enzyme

lized ligand in the vicinity of the active site of the enzyme with energy score (S) = -22.6799 Kcal/mol and root mean standard deviation (RMSD) = 0.8205 (Fig. 1). The ligand interacts with the active site amino acids by three interactions: with Met 343 with hydrogen bond of 3.09 Å, with Glu 341 with hydrogen bond of 2.22 Å and with Thr 340 with hydrogen bond of 3.22 Å.

All the synthesized compounds were docked on the active site of the enzyme showing good fitting. The energy score (S) as well as amino acid interactions of the synthesized compounds are listed in Table 1.

The best energy scores were exhibited by compounds **7g** and **7l** with $S = -20.4112$ and -22.8663 Kcal/mol, respectively. Figures 2 and 3 describe the amino acid interactions with compounds **7g** and **7l**, respectively.

In vitro antitumor activity

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against

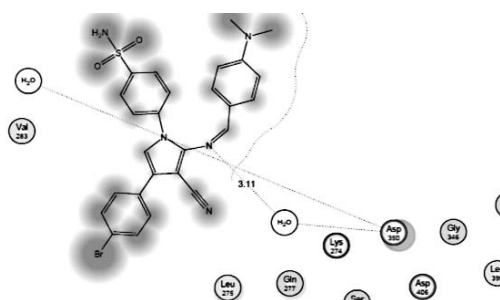


Figure 2. Compound **7g** on the active site of c-Src enzyme

human breast cancer cell line (MCF7). Doxorubicin, which is one of the most effective anticancer agents, was used as the reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of breast cancer cell line (MCF7). The response parameter calculated was the IC_{50} value, which corresponds to the concentration required for 50% inhibition of cell viability. *In vitro* cytotoxic activity of the synthesized compounds compared to the reference drug is presented in Table 2.

The strategic starting material – pyrrole derivative **5** – showed IC_{50} value 7.65 μM which did not change upon cyclization to the amino pyrrolopyrimidine derivative **6**. However, the formation of several Schiff's bases **7a–m** dramatically influenced the activity, especially for compounds **7a**, **7e**, **7g**, **7i**, **7j**, and **7l**, with IC_{50} values of 6.74 , 6.47 , 6.46 , 6.46 , 6.74 and 6.70 μM , respectively. On the other hand, the pyrrolopyrimidine derivative **8** showed good IC_{50} value of 6.74 μM . In case of the trifluoroacetyl derivative **9**, the IC_{50} value was 7.84 μM while the monoacetyl pyrrolo derivative **10** showed IC_{50} value of 7.56 μM , which was better for the diacetyl analogue **11** with IC_{50} value of 6.74 μM . The IC_{50} values of 6.29 μM were observed for the mono and dichloroacetyl derivatives **13** and **14**, respectively. Finally, all the synthesized compounds showed better IC_{50} than doxorubicin except compounds **7k** and **7m** with IC_{50} values of 8.15 and 8.45 μM , respectively. Compounds **7g** and **7l** showed both good IC_{50} of 6.46 and 6.70 μM and also good docking score of -20.4112 and -22.8663 Kcal/mol, suggesting good candidates for c-Src inhibitors.

CONCLUSION

The objective of the present study was to synthesize and investigate the anticancer activity of some novel pyrrolo and pyrrolopyrimidine deriva-

tives carrying the biologically active sulfonamide moieties. Most of the synthesized compounds showed good activity with better IC₅₀ than doxorubicin as reference drug, especially compounds **7a**, **7e**, **7g**, **7i**, **7j**, **8** and **11**. Also, compounds **5**, **6**, **7b-d**, **7f**, **7h**, **7l**, **9**, **10**, **13**, **14** are nearly as active as doxorubicin as positive control, while compounds **7k** and **7m** showed activity lower than doxorubicin.

Acknowledgment

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UTILITY OF L-NOREPHEDRINE IN THE SEMISYNTHESIS OF NOVEL THIOUREA AND THIAZOLIDINE DERIVATIVES AS A NEW CLASS OF ANTICANCER AGENTS

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Abstract: The natural alkaloid l-norephedrine **1** was utilized in the synthesis of some novel thiourea derivatives **2**, **5** and thiazolidinones **4a,b** and **6**, **7**. Structures of the synthesized compounds were confirmed by analytical and spectral data. The synthesized compounds were evaluated *in vitro* for anticancer activity against the human breast (MCF-7), human liver (HEPG2) and human colon (HCT116) cancer cell lines. Thiazolidinone derivative **7** was the most active against all the cell lines with values $IC_{50} = 2.60, 2.80$ and $2.60 \mu\text{g/mL}$ compared with doxorubicin ($IC_{50} = 5.40, 2.97$ and $5.26 \mu\text{g/mL}$). Thiazolidinone derivative **6** exhibited higher activity with IC_{50} value ($3.20 \mu\text{g/mL}$) against HCT116 when compared with doxorubicin with IC_{50} value ($5.26 \mu\text{g/mL}$) as positive control.

Keywords: l-norephedrine, thiourea, oxazolidine, thiazolidinones, anticancer activity

Most cancer patients are subjected to chemotherapy for the treatment of advanced cancers. However, most metastatic solid tumors eventually remain incurable even by treatment with recent anticancer drugs. Also, cancer is a disease of striking significance in the world today. It is the second leading cause of death in the world after cardiovascular diseases and it is projected to beginning the primary cause of death there within the coming years (1, 2). The identification of novel structures that can be potentially useful in designing new, potent selective and less toxic anticancer agents is still a major challenge to medicinal chemistry researchers (3). Despite of the important advances achieved over recent decades in the research and development of various cancerostatic drugs, current antitumor chemotherapy still suffers from two major limitations - the first is the lack of selectivity of conventional chemotherapeutic agents for cancer tissues, bringing about unwanted side effects. The sec-

ond is the acquisition by cancer cells of multiple-drug resistance. Unwanted side effects of antitumor drugs could be overcome with agents capable of discriminating tumor cells from normal proliferative cells and the resistance is minimized using combined modality approach with different complementary mechanism of action (4). The current scenario highlights the need for the discovery and development of new lead compounds of simple structure, exhibiting optimal *in vivo* antitumor potency and new mechanisms of action. Recent advances in clinical techniques, including large cooperative studies, are allowing more rapid and reliable evaluation of new drugs. The combination of these advantages with improved preliminary screening systems is enhancing the emergence of newer and more potent compounds. In this regard, it should be emphasized that National Cancer Institute (NCI) *in vitro* primary anticancer drug screen represents a valuable research tool to facilitate the drug discovery of new

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structural/mechanistic types of antitumor agents (5). The main sources of lead compounds for drug development are natural products, new synthetic compounds and analogs of new agents (6). The thiourea derivatives (**I**) (7–9) represent one of the generally most useful classes of anticancer agents (Fig. 1). In addition, thiazolidinone template is a privileged structure fragments in modern medicinal chemistry considering its broad pharmacological spectrum and affinity for various biotargets of these class heterocyclic compounds. It is among the usually occurred heterocyclic nuclei in many marine as well as natural plant products possessing wide range of biological applications (10–12). On the other hand, thiazolidinone derivatives are well known class of biological active substances (13–15) that became basic for the whole number of innovative medicinal agents, such as hypoglycemic thiazolidinediones (pioglitazone and its analogs) (16), aldose reductase inhibitors (epalrestat) (17), dual inhibitors of COX-2/5-LOX (darbufelon) (18), modern diuretics. (etozoline) (19), Mur family inhibitors (UDP-MurNAc/L-Ala ligases) (20). Recently, thiazolidinones research area unexpectedly became interesting and promising for oncology. In depth study of PPARs allowed to put forward and validate the concept of anticancer potential existence of PPAR agonists including thiazolidinediones (21, 22). In addition, inhibitors of antiapoptotic proteins Bcl-XL and BH3 (23), which contribute to modulation of programmed cell death (apoptosis), as well as inhibitors

of tumor necrosis factor TNF α (24), necroptosis inhibitors (25), integrin antagonists (26), inhibitors of JSP-1 (27), Pim-2 and Pim-1 protein kinases (28), COX-2 (29) were identified among 4-thiazolidinones. Figure 1 presents «hit-compounds» (**I** – **IV**) from different groups (30, 31) that possess high antimitotic effect *in vitro* in submicromolar concentrations (10, 5, 10 and 7 M, respectively) and are characterized by the low *in vivo* toxicity level.

Since 1990, we have been working on the synthesis of polycyclic systems containing quinoline, quinazoline and thienopyrimidine nucleus with a biologically active sulfonamide moiety in order to test their anticancer and radiosensitizing activities (32–38). In the light of these facts, and as a continuation of reported work (39, 40), we planned to synthesize novel thiourea and thiazolidinone derivatives by using 1-norephedrine (phenylpropanolamine) **1** as starting material to evaluate their anticancer activities hoping to obtain compounds with significant anticancer potential.

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 are uncorrected. Elemental analyses (C, H, N) were per-

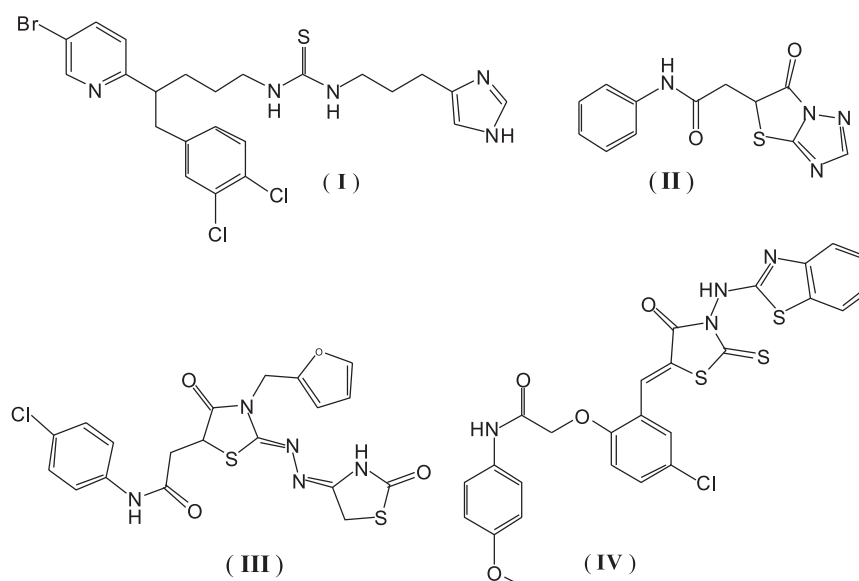


Figure 1. Structures of some anticancer lead compounds from the literature

formed on a Perkin-Elmer 2400 Instrument (USA). All results were within $\pm 0.4\%$ of the theoretical values. Infrared (IR) spectra (KBr disc) were recorded on FT-IR spectrophotometer (Perkin Elmer) at the Research Center, College of Pharmacy, King Saud University, Saudi Arabia. ^1H and ^{13}C NMR spectra were recorded on a UltraShield Plus 500 MHz (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) relative 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).

1-Ethyl-3-(1-hydroxy-1-phenylpropan-2-yl)thiourea (2) and 4-methyl-5-phenyloxazolidine-2-thione (3b)

L-Norephedrine (151 mg, 1 mmol) was added to a solution of ethyl isothiocyanate (87 mg, 1 mmol) dissolved in chloroform (20 mL) containing triethylamine (101 mg, 1 mmol). The reaction mixture was stirred for 5 min at room temperature. The solution was evaporated to dryness under reduced pressure and chromatographed on silica gel column (2 mm i.d., 30 g) eluted with dichloromethane. Polarity was increased with methanol in a gradient system. Fractions of 50 mL were collected, screened by TLC and similar fractions were pooled. Fractions 3–5 afforded 26 mg of **3b**. Fractions 8–10 were further purified on silica gel PTLC using $\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95 : 5 (v/v) as developing system to yield 135 mg of **2**.

2: Yield 68%; semisolid; IR (KBr, cm^{-1}): 3483 (OH), 3387, 3219 (2NH), 3087 (CH arom.), 2977, 2836 (CH aliph.), 1287 (C=S). ^1H -NMR (500 MHz, DMSO-d_6 , δ , ppm): 0.86 (d, $J = 6.7$ Hz, 3H, CH_3 of l-norephedrine), 1.08 (t, $J = 7.2$ Hz, 3H, $\text{NH-CH}_2\text{-CH}_3$), 3.41 (NH- $\text{CH}_2\text{-CH}_3$, signal partially obscured by H_2O signal), 4.47 (bs, 1H, NH-CH- of l-norephedrine), 4.87 (bs, 1H, CH-OH), 5.47 (bs, 1H, CH-OH), 7.22–7.52 (m, 6H, aromatic and NH of l-norephedrine), 7.46 (t, $J = 4.5$ Hz, 1H, $\text{NH-CH}_2\text{-CH}_3$). ^{13}C -NMR (125 MHz, DMSO-d_6 , δ , ppm): 12.68, 14.42, 38.77, 54.72, 73.43, 125.69–127.81 (5), 143.49, 181.31. MS m/z (%): 238 (12.8, M^+). Analysis: calcd. for $\text{C}_{12}\text{H}_{18}\text{N}_2\text{OS}$: C, 60.47; H, 7.61; N, 11.75%; found: C, 60.12; H, 7.39; N, 11.98%.

3b: Yield 29%, m.p. 90–92°C, IR (KBr, cm^{-1}): 3280 (NH), 3099 (CH arom.), 2956, 2871 (CH aliph.), 1280 (C=S). ^1H -NMR (500 MHz, DMSO-d_6 ,

δ , ppm): 0.65 (d, $J = 5.8$ Hz, 3H, CH_3), 4.42 (bs, 1H, NH-CH-CH_3), 6.00 (d, $J = 8.5$ Hz, 1H, -CH-O), 7.25–7.42 (m, 5H, arom.), 10.20 (bs, 1H, NH). ^{13}C -NMR (125 MHz, DMSO-d_6 , δ , ppm): 16.04, 54.89, 84.87, 126.10–128.41 (5), 134.82, 187.44. MS m/z (%): 193 (96.0, M^+). Analysis: calcd. for $\text{C}_{10}\text{H}_{11}\text{NOS}$: C, 62.15; H, 5.74; N, 7.25%; found: C, 62.44; H, 5.51; N, 7.61%.

2-(Ethylimino)-3-(1-hydroxy-1-phenylpropan-2-yl)thiazolidin-4-one (4a) and 3-ethyl-2-(1-hydroxy-1-phenylpropan-2-ylimino)thiazolidin-4-one (4b)

A solution of chloroacetyl chloride (70 mg, 0.6 mmol) in 15 mL of chloroform was added dropwise to a stirred solution of **2** (119 mg, 0.5 mmol) and triethylamine (60 mg, 0.6 mmol). The reaction was stirred overnight and then the mixture was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column (2 mm i.d., 20 g) eluted with dichloromethane. Polarity was increased with methanol in a gradient system. Fractions 4–7 afforded 7 mg of **4a**, while fractions 9–15 afforded 54 mg of **4b**.

4a: Yield 19%; semisolid, IR (KBr, cm^{-1}): 3455 (OH), 3100 (CH arom.), 2960, 2848 (CH aliph.), 1699 (C=O), 1612 (C=N). ^1H -NMR (500 MHz, CDCl_3 , δ , ppm): 1.21 (m, 6H, $2\times\text{CH}_3$), 3.34 (bs, 2H, $\text{S-CH}_2\text{-C=O}$), 3.88 (q, $J = 8.4$ Hz, 2H, $\text{N-CH}_2\text{-CH}_3$), 4.80 (bs, 1H, N-CH- of l-norephedrine), 5.02 (bs, 1H, CH-OH), 7.26–7.46 (m, 5H, arom.). ^{13}C -NMR (125 MHz, CDCl_3 , δ , ppm): 12.26, 15.49, 32.66, 46.62, 58.65, 75.05, 126.05–128.32 (5), 142.06, 171.51. MS m/z (%): 278 (26.2, M^+). Analysis: calcd. for $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_2\text{S}$: C, 60.41; H, 6.52; N, 10.06%; found: C, 60.18; H, 6.88; N, 10.27%.

4b: Yield 61%; semisolid, IR (KBr, cm^{-1}): 3412 (OH), 3068 (CH arom.), 2969, 2855 (CH aliph.), 1702 (C=O), 1608 (C=N). ^1H -NMR (500 MHz, CDCl_3 , δ , ppm): 0.98 (m, 6H, $2\times\text{CH}_3$), 3.45 (bs, 1H, NH-CH-CH_3), 3.60 (m, 2H, $2\times\text{CH}_2$), 4.47 (bs, 1H, N-CH- of l-norephedrine), 4.61 (bs, 1H, CH-OH), 4.87 (bs, 1H, CH-OH), 7.17–7.27 (m, 5H, arom.). ^{13}C -NMR (125 MHz, CDCl_3 , δ , ppm): 12.28, 15.15, 32.64, 37.98, 63.16, 77.19, 126.61–128.14 (5), 141.21, 151.65, 171.44. MS m/z (%): 278 (18.3, M^+). Analysis: calcd. for $\text{C}_{14}\text{H}_{18}\text{N}_2\text{O}_2\text{S}$: C, 60.41; H, 6.52; N, 10.06%; found: C, 60.67; H, 6.28; N, 9.71%.

1-Cyclohexyl-3-(1-hydroxy-1-phenylpropan-2-yl) thiourea (5)

L-Norephedrine **1** (151 mg, 1 mmol) was added to a solution of cyclohexyl isothiocyanate (141 mg,

1 mmol) dissolved in chloroform (20 mL) containing triethylamine (101 mg, 1 mmol). The reaction mixture was stirred for 10 min at room temperature. The solution was evaporated to dryness under reduced pressure and loaded on the top of silica gel column (2 mm i.d., 30 g) eluted with dichloromethane. Fractions of 50 mL were collected, screened by TLC and similar fractions were pooled. Fractions 3–6 afforded 170 mg of **5**.

5: Yield 93%; m.p. 124–125°C, IR (KBr, cm⁻¹): 3446 (OH), 3327, 3276 (2NH), 3088 (CH arom.), 2949, 2868 (CH aliph.), 1288 (C=S). ¹H-NMR (500 MHz, DMSO-d₆, δ, ppm): 0.84 (d, *J* = 6.7 Hz, 3H), 1.16–1.86 (m, 10H), 4.01 (bs, 1H, CH- cyclohexyl), 7.21–7.42 (m, 7H, arom., 2NH), 4.47 (bs, 1H, CH-NH l-norephedrine); 4.87 (bs, 1H, CH-OH), 5.50 (bs, 1H, CH-OH). ¹³C-NMR (125 MHz, DMSO-d₆, δ, ppm): 12.51, 24.35 (2), 25.19, 32.34 (2), 51.48, 54.57, 73.36, 125.63–127.83 (5), 143.54, 181.57. MS *m/z* (%): 292 (6.4, M⁺). Analysis: calcd. for C₁₆H₂₄N₂O₂S: C, 65.71; H, 8.27; N, 9.58%; found: C, 65.48; H, 8.52; N, 9.26%.

2-(Cyclohexylimino)-3-(1-hydroxy-1-phenylpropan-2-yl)-thiazolidin-4-one (6) and 2-(3-cyclohexyl-4-oxothiazolidin-2-ylideneamino)-1-phenylpropyl-2-chloroacetate (7)

A solution of chloroacetyl chloride (70 mg, 0.6 mmol) in chloroform (15 mL) was added dropwise to a stirred solution of **5** (146 mg, 0.5 mmol) and triethylamine (60 mg, 0.6 mmol). The reaction was stirred overnight and then the reaction mixture was evaporated to dryness under reduced pressure. The residue was chromatographed on a silica gel column (2 mm i.d., 30 g) eluted with dichloromethane. Polarity was increased with methanol in a gradient

system. Fractions 3–5 afforded 94 mg of **7**, while fractions 7–11 afforded 35 mg of **6**.

6: Yield 23%; semisolid, IR (KBr, cm⁻¹): 3493 (OH), 3094 (CH arom.), 2944, 2863 (CH aliph.), 1689 (C=O), 1598 (C=N). ¹H-NMR (500 MHz, DMSO-d₆, δ, ppm): 1.16 (d, *J* = 6.5 Hz, 3H), 1.17–1.72 (m, 10H), 3.08 (bs, 1H, CH- cyclohexyl), 3.79 (q, *J* = 10.5 Hz, 2H, O=C-CH₂-S), 4.71 (d, *J* = 5.5 Hz, 1H, CH-NH l-norephedrine); 4.94 (bs, 1H, CH-OH), 7.17–7.37 (m, 5H, arom.). ¹³C-NMR (125 MHz, DMSO-d₆, δ, ppm): 9.16, 24.37 (2), 25.49, 33.13 (2), 33.37, 58.60, 61.29, 75.11, 126.06–128.20 (5), 142.01, 169.98. MS *m/z* (%): 332 (18.5, M⁺). Analysis: calcd. for C₁₈H₂₄N₂O₂S: C, 65.03; H, 7.28; N, 8.43%; found: C, 64.76; H, 7.54; N, 8.77%.

7: Yield 74%; semisolid, IR (KBr, cm⁻¹): 3079 (CH arom.), 2981, 2836 (CH aliph.), 1710, 1688 (2C=O), 1618 (C=N), 786 (C-Cl). ¹H-NMR (500 MHz, DMSO-d₆, δ, ppm): 1.08 (d, *J* = 6.2 Hz, 3H), 1.17–1.71 (m, 10H), 3.57 (d, *J* = 5.5 Hz, 1H, CH-NH l-norephedrine), 4.06 (m, 4H, O=C-CH₂-S and Cl-CH₂-C=O), 4.94 (bs, 1H, CH- cyclohexyl), 5.76 (bs, 1H, CH-OH), 7.17–7.30 (m, 5H, arom.). ¹³C-NMR (125 MHz, DMSO-d₆, δ, ppm): 17.13, 24.35 (2), 25.70, 32.37 (2), 33.47, 41.05, 53.10, 55.65, 61.33, 80.77, 127.62–128.81 (5), 136.44, 166.35, 171.61. MS *m/z* (%): 408 (15.2, M⁺). Analysis: calcd. for C₂₀H₂₅ClN₂O₃S: C, 58.74; H, 6.16; N, 6.85%; found: C, 58.64; H, 6.51; N, 6.49%.

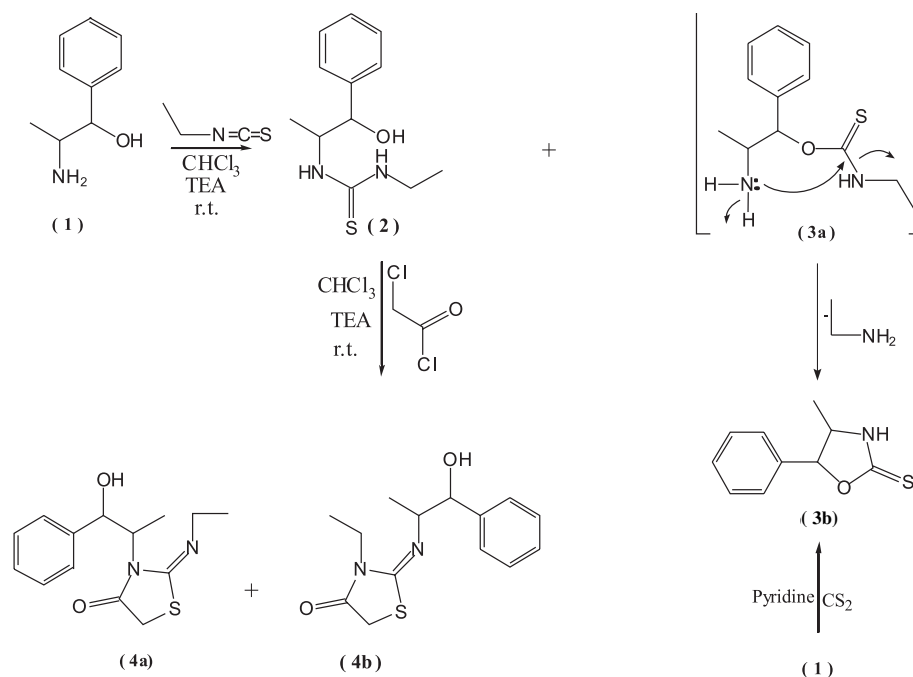
In vitro antitumor activity

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. (41). The *in vitro* anticancer screen-

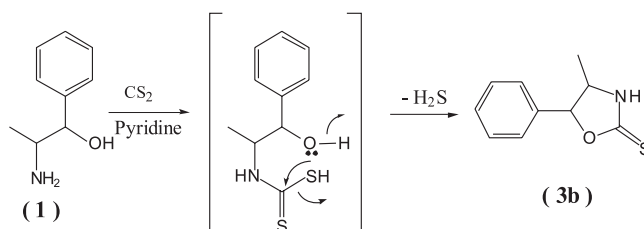
Table 1. *In vitro* anticancer screening of the newly synthesized compounds against human breast (MCF-7), liver (HEPG2), and colon (HCT 116) cancer cell lines.

Compd. No.	IC ₅₀ (µg/mL) ^a		
	MCF-7	HEPG2	HCT116
Doxorubicin	5.40	2.97	5.26
2	24.00	36.20	40.80
3b	43.00	37.40	41.80
4a	NA	NA	NA
4b	34.80	32.80	27.00
5	41.40	28.60	25.00
6	6.80	NA	3.20
7	2.60	2.80	2.60

^a: IC₅₀ value: Concentration causing 50% inhibition of cell viability. NA = No activity.



Scheme 1. Synthesis of oxazolidine and thiazolidinone derivatives



Scheme 2. Postulated mechanism for the formation of compound 3b

ing was done at the Pharmacology Unit, the National Cancer Institute, Cairo University. Cells were plated in 96 multiwell microtiter plate (104 cells/well) for 24 h before treatment with the compound(s) to allow attachment of cell 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 $\mu\text{g}/\text{mL}$) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) 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. Excess 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 (41). 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). The response parameter calculated was IC_{50} value, which corresponds to the

concentration required for 50% inhibition of cell viability.

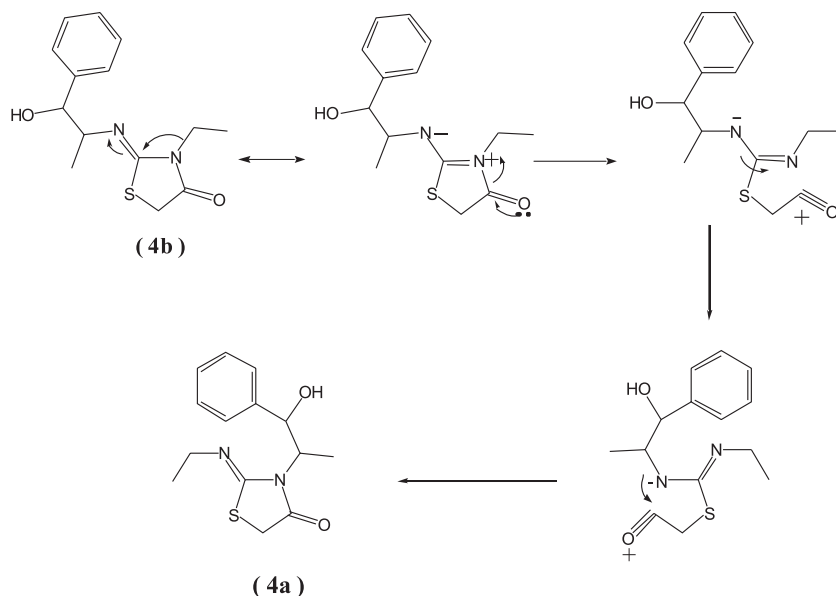
RESULTS AND DISCUSSION

Chemistry

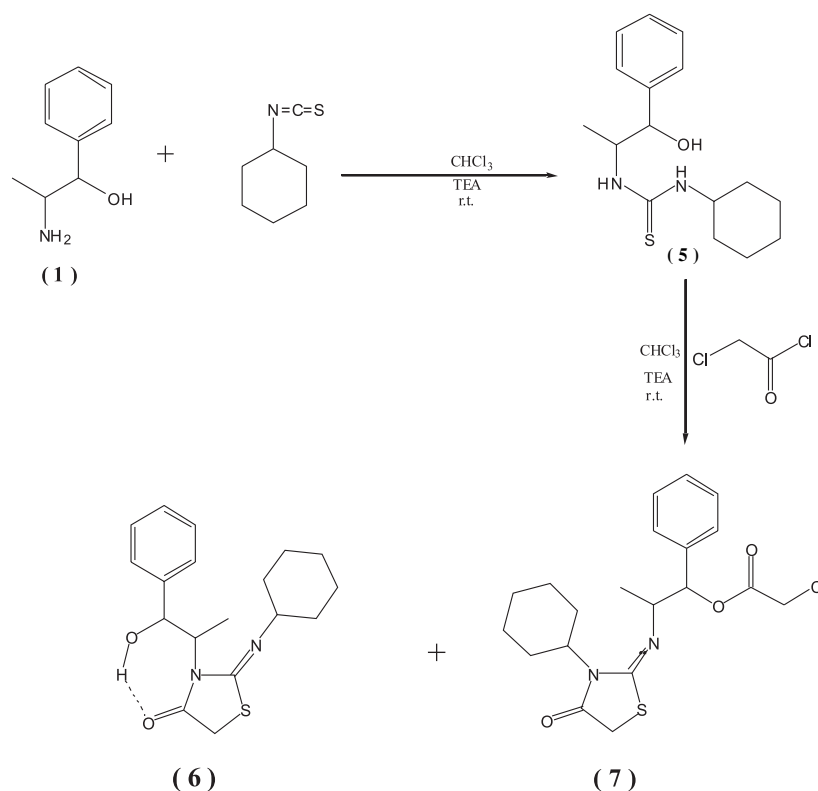
The compounds were designed in order to explore their anticancer activity. The behavior of the natural alkaloid 1-norephedrine **1** with both nitrogen and oxygen nucleophiles toward isothiocyanate was studied. Thus, when **1** (2-amino-1-phenylpropan-1-ol) was allowed to react with ethyl isothiocyanate in chloroform in the presence of triethylamine as catalyst at room temperature for 5 min, the corresponding 1-ethyl-3-(1-hydroxy-1-phenylpropan-2-yl)-thiourea (**2**) and 4-methyl-5-phenyloxazolidine-2-thione **3b** (**42**) were obtained (Scheme 1). Compound **3b** was produced *via* initial formation of intermediate **3a** followed by intermolecular cyclization with elimination of ethylamine (Scheme 1, 2). Also, **3b** was obtained in one step under different conditions *via* reaction of **1** with carbon disulfide in pyridine (m.p. = 90–92°C as reported) (Scheme 1). The structures of **2** and **3b** were elucidated from elemental analysis and spectral data. The IR spectra of **2** showed the absence of NH₂ bands and the presence of characteristic bands for OH, NH and C=S. Its ¹H-NMR spectrum showed $\text{CH}_3\text{-CH}_2\text{-NH}$ of ethyl isothiocyanate, which appeared at δ_{H} 1.07 (t) ppm, δ_{C} 14.42 ppm, $\text{CH}_3\text{-CH}_2\text{-NH}$ δ_{H} 3.40 (m) ppm, δ_{C}

38.18, $\text{CH}_3\text{-CH}_2\text{-NH}$ δ_{H} 7.46 (t) ppm. These assignments were based on COSY, HSQC and HMBC experiments. Further support for the structure of **2** was the carbon signal at δ_{C} 181.49 ppm assigned for the C=S carbon. The secondary alcohol group of 2-amino-1-phenylpropan-1-ol showed signals at δ_{H} 4.87 (bs) ppm and δ_{C} 73.44 ppm in ¹H- and ¹³C NMR, respectively. The hydroxyl proton was assigned to the signal at δ_{H} 5.47 (bs) ppm based on the COSY correlation with the CH-O proton at δ_{H} 4.87 ppm. The oxazolidine-2-thione moiety in **3b** was evident from signals at δ_{H} 6.01 (d); δ_{C} 84.87 (CH-O-), δ_{H} 4.42 (bs); δ_{C} 54.89 (CH-NH) and δ_{H} 10.20 (bs) ppm (CH-NH) and the disappearance of the OH signal.

Subsequent synthesis of 2-imino-4-thiazolidinones **4a,b** was performed by condensation of thiourea **2** with chloroacetyl chloride in the presence of triethylamine in CHCl₃ at room temperature. The reaction provided a mixture of two isomers: ethylimino **4a** and hydroxyphenylpropaneimino **4b** in about 1 : 3 ratio after 15 min. The ratio was maintained when the reaction was left overnight and monitored by TLC. They conceivably originated from the condensation of chloroacetyl chloride with the sulfur atom of two different intermediate thiols generated from **2** by delocalization of the lone pairs of the two different nitrogen atoms on the adjacent thiocarbonyl group. The synthesis of the isomer **4b** was favored by the intermediate thiol involving the NH group adjacent to the electron releasing group.



Scheme 3. Rearrangement of **4b** into **4a**



Scheme 4. Formation of thiourea and thiazolidinone derivatives

On the other hand, when the reaction was performed in MeOH at reflux, both isomers were present with notable decrease of **4b** by time based on TLC study. After 24 h, the most stable isomer **4a** was the only product indicating that **4b** converted to **4a**. The suggested mechanism of the conversion of **4b** into **4a** is depicted in Scheme 3. The extended electronic delocalization of amidine system gave rise to the cleavage of the cyclic amide bond. A possible low barrier around the C-S σ bond and the subsequent cyclization accounted for the intermolecular rearrangement providing the most stable compound **4a**.

The structures of **4a,b** were supported based on elemental analysis, IR, ^1H -, ^{13}C -NMR and mass spectral data. The IR spectrum of **4a** exhibited the absence of NH band and the presence of characteristic bands for OH and C=O. In both compounds, the groups adjacent to the newly formed C=N resonate at different chemical shifts due to the increased deshielding effect generated by the extended electronic delocalization of the N lone pair. The ^1H - and ^{13}C -NMR spectra of **4a** indicated that the resonances of the $-\text{CH}_2\text{-N}-$ group of ethyl isothiocyanate was shifted to δ_{H} 3.88 (q) and δ_{C}

46.62 ppm. In **4b**, the shift was more evident at the 2-amino-1-phenylpropan-1-ol moiety. The $=\text{N-CH}(\text{CH}_3)\text{-CH-OH}$ signals appeared at δ_{H} 3.45; δ_{C} 63.16 ($=\text{N-CH}$), δ_{H} 0.98 (d); δ_{C} 12.28 (CH_3), δ_{H} 4.61 (bs); δ_{C} 77.13 (CH-OH) ppm. On the other hand, when **1** was allowed to react with cyclohexyl isothiocyanate in CHCl_3 in the presence of triethylamine as catalyst at room temperature for 15 min, the corresponding 1-cyclohexyl-3-(1-phenylpropan-2-yl)thiourea **5** was obtained in good yield (Scheme 4). Structure of compound **5** was supported on the basis of elemental analysis, IR, ^1H -, ^{13}C -NMR and mass spectral data. Its IR spectrum exhibited the absence of NH_2 band, and the presence of a characteristic bands for NH, OH and C=S. ^1H -NMR showed signals at δ_{H} 7.21–7.42 ppm corresponding for five aromatic protons and δ_{H} 1.16–1.86 ppm assigned to ten cyclohexyl protons. The $\underline{\text{CH}}$ - of cyclohexyl appeared at δ_{H} 4.01 (bs); δ_{C} 51.48 ppm, while that of 2-amino-1-phenylpropan-1-ol appeared at δ_{H} 4.47 (bs); δ_{C} 54.57 ppm in both ^1H - and ^{13}C NMR. The CH- of cyclohexyl appeared at δ_{H} 7.34, while that of 2-amino-1-phenylpropan-1-ol appeared at δ_{H} 7.23 ppm. These assignments were based on COSY, HSQC and HMBC experiments.

Further support for the thiourea derivative **5** was the carbon signal at δ_c 181.57 ppm assigned for the C=S carbon. One of the characteristic groups of the structure is the secondary alcohol group of 2-amino-1-phenylpropan-1-ol showed signals at δ_H 4.87 (bs); δ_c 73.36 ppm in both 1H - and ^{13}C -NMR spectra. The hydroxyl proton was assigned to the signal at δ_H 5.50 (bs) ppm based on the COSY correlation with the \underline{CH} -O proton at δ_H 4.87 ppm. Subsequent synthesis of 2-imino-4-thiazolidinones **6**, **7** was performed by condensation of thiourea **5** with chloroacetyl chloride in chloroform in the presence of triethylamine at room temperature (Scheme 4). The structure of isomers **6** and **7** was assessed by elemental analysis, IR, 1H - and ^{13}C -NMR. In compound **6**, the resonances of the \underline{CH} -NH of cyclohexyl was shifted to δ_H 3.08 (bs); δ_c 60.23 ppm. The hydroxyl proton appears at δ_H 4.94 (bs) based on its COSY correlation with the \underline{CH} -O proton at δ_H 4.71 ppm. In compound **7**, the shifts were more evident at the 2-amino-1-phenylpropan-1-ol moiety. The =N-CH(CH₃)-CH-OH signals appeared at δ_H 3.57; δ_c 61.33 (=N-CH), δ_H 1.08 (d); δ_c 17.13 (CH₃), δ_H 5.57 (bs); δ_c 80.77 (CH-OH) ppm. In both compounds the groups adjacent to the newly formed C=N resonate at different chemical shift due to the increased deshielding effect generated by the extended electronic delocalization of the N lone pair. In the spectrum of **7** the disappearance of the OH proton, the down field shift of the \underline{CH} -OH, the additional CH₂ signal at δ_H 4.06; δ_c 41.05 ppm and the carbonyl signal at δ_c 166.31 ppm were diagnostic for further acylation of the OH group. The inability of the hydroxyl group in **6** to react with chloroacetyl chloride under the same conditions as compound **7** is most likely due to the formation of hydrogen bonding with the carbonyl of thiazolidinone ring (Scheme 4).

***In vitro* antitumor activity**

The newly synthesized compounds were evaluated for their *in vitro* cytotoxic activity against human breast cancer cell line (MCF-7), human liver cancer cell line (HEPG2) and human colon cancer cell line (HCT 116). Doxorubicin, which is one of the most effective anticancer agents, was used as the reference drug in this study. The relationship between surviving fraction and drug concentration was plotted to obtain the survival curve of cancer cell lines. The response parameter calculated was the IC₅₀ value, which corresponds to the concentration required for 50% inhibition of cell viability. Table 1 shows the *in vitro* cytotoxic activity of the synthesized compounds, where the thiazolidi-

none derivative **7** having the cyclohexyl moiety at 3-position with 1-hydroxyl-1-phenylpropane-imino moiety at 2-position was the most active compound against all cancer cell lines with IC₅₀ values (2.60, 2.80, 2.60 μ g/mL) compared with the doxorubicin with IC₅₀ value (5.40, 2.97, 5.26 μ g/mL). On the other hand, thiazolidinone derivative **6** carrying the cyclohexylimino moiety at 2-position with 1-hydroxy-1-phenylpropane at 3-position exhibited higher activity against the HCT 116 with IC₅₀ value (3.20 μ g/mL) compared with doxorubicin with IC₅₀ value (5.26 μ g/mL) and moderate activity less than doxorubicin with IC₅₀ value (6.80 μ g/mL) against MCF-7.

CONCLUSION

The objective of the present study was to semi-synthesize and investigate the anticancer activity of some novel thiourea and thiazolidinone derivatives carrying the biologically active cyclohexyl and cyclohexylimino moieties. Compounds **7** showed promising anticancer activity higher than that of doxorubicin as reference drug against all the tested cancer cell lines, while compound **6** exhibited higher activity against colon cancer cell line and more active than that of doxorubicin. Also, compound **6** is nearly as active as doxorubicin as positive control against breast cancer cell line.

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NATURAL DRUGS

PHOSPHOLIPASES A₂: ENZYMATIC ASSAY FOR SNAKE VENOM
(*NAJA NAJA KARACHIENSIS*) WITH THEIR NEUTRALIZATION
BY MEDICINAL PLANTS OF PAKISTAN

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Abstract: Phospholipases A₂ (PLA₂) are the most lethal and noxious component of *Naja naja karachiensis* venom. They are engaged to induce severe toxicities after their penetration in victims. Present study was designed to highlight hydrolytic actions of PLA₂ in an egg yolk mixture and to encounter their deleterious effects via medicinal plants of Pakistan. PLA₂ were found to produce free fatty acids in a dose dependent manner. Venom at concentration of 0.1 mg was found to liberate 26.6 μmoles of fatty acids with a decline in pH of 0.2 owing to the presence of PLA₂ (133 Unit/mg). When quantity of venom was increased up to 8 mg, it caused to release 133 μmoles of free fatty acids with a decrease in 1.0 pH due to abundance in PLA₂ (665 Unit/mg). The rest of other doses of venom (0.3–4.0 mg) was found to liberate fatty acids between these two upper and lower limits. Twenty eight medicinal plants (0.1–0.6 mg) were tried to abort PLA₂ hydrolytic action, however, all were found useful (50–100%) against PLA₂. *Bauhinia variegata* L., *Citrus limon* (L.). *Burm. f.*, *Enicostemma hyssopifolium* (Willd.) Verdoorn, *Ocimum sanctum*, *Psoralea corylifolia* L. and *Stenolobium stans* (L.) D. Don were found excellent in switching off 100% phospholipases A₂ at their lowest concentration (0.1 mg). Three plants extract were found useful only at lower concentration (0.1 mg), however, their higher doses were seemed to aggravate venom response. Eight medicinal plants failed to neutralize PLA₂ rather their higher doses were found effective. Standard antidote and rest of other plants extract were able to show maximum of 50% efficiencies. Therefore, it is necessary to identify and isolate bioactive constituent(s) from above cited six medicinal plants to eradicate the problem of snake bite in the future.

Keywords: phospholipases A₂, Pakistan cobra, medicinal plants, acidimetric assay, antidote

Fright from snakes is as primitive as human history. Perhaps snake biting is the most neglected tropical disease, which affects 2.5 million people and results in 100 000 deaths annually (1). There are more than two hundred species of the snakes, which are venomous on the earth. They belong to polyphyletic group of Colubroidea, however, can be classified into Cortalidae, Elapidae, Hydrophidae and Viperidae families (2, 3). Elapidae belonging to genus *Naja* are quite ubiquitous and constitute ten full species of Asiatic *Naja*. Among them, black

Pakistan cobra (*Naja naja karachiensis*) is deadly poisonous and considered a sign of threat particularly in southern Punjab province of Pakistan (4, 5).

In fact, snake venoms are complex mixture of various enzymatic (hydrolytic), non-enzymatic, inorganic and organic molecules. Among hydrolytic enzymes, phospholipases A₂ are present in copious quantity, therefore their contribution towards detrimental effects cannot be waived (6). Owing to their diverse actions (mostly on the site of action) they were classified into phospholipases A₁ (hydrolyze 1-acyl group),

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phospholipases A₂ (hydrolyze central and 2-acyl group) phospholipases C and phospholipases D that hydrolyze phosphodiester linkages (7–9). Phospholipases A₂ have been responsible for many pathophysiological disorders like cardiotoxicity, neurotoxicity, edema, necrosis, hemolysis, amputation and anti-coagulation. Moreover, generation of free radicals along with reactive oxygen produced toxicities of phospholipases in the victims of snake bite (10, 11).

To neutralize deleterious effects of these phospholipases (PLA₂) numerous enzyme inhibitors have been tested previously to abstain from their

undesired effects. Among them, natural antidotes (medicinal plants) have been considered the most reliable source to neutralize snakes venom. Pakistan is the hub of medicinal flora where people rely on medicinal plants for their health-related problems particularly to treat snakebite (12). It was therefore, inevitable to prove scientifically folklore claims about these medicinal plants as anti-snake venom.

To bridge this gap, present research work was designed to rationalize scientifically medicinal plants of Pakistan against *Naja naja karachiensis* phospholipases induced toxicities. It includes plants

Table1. Complete description about Pakistani medicinal plants collected for evaluation of their potentials as anti-snake venom.

No.	Tested sample (medicinal plants)	Part collected	References (anti-venom)
1	<i>Albizia lebbbeck</i> (L.) Benth.	Seeds	18
2	<i>Allium cepa</i> L.	Bulb	19
3	<i>Allium sativum</i> L.	Bulb	20
4	<i>Althaea officinalis</i> L.	Roots	12
5	<i>Bauhinia variegata</i> L.	Roots	21
6	<i>Brassica nigra</i> (L. Koch)	Seeds	18
7	<i>Calotropis procera</i> (Wild.) R.Br.	Exudates Flowers	12
8	<i>Cedrus deodara</i> G. Don	Bark	18
9	<i>Citrus limon</i> (L.) Burm. f	Fruit	22
10	<i>Citrullus colocynthis</i> Schard.	Fruits	18
11	<i>Cuminum cyminum</i> L	Seeds	18
12	<i>Encostemma hyssopifolium</i> (Willd.) Verdoorn	Full plant	23
13	<i>Fagonia cretica</i> L.	Leaves	13
14	<i>Leucas capitata</i> Desf.	Full plant	21
15	<i>Matthiola incana</i> (L.) R.Br.	Seeds	18
16	<i>Momordica charantia</i> L.	Fruit	18
17	<i>erium indicum</i> Mill.	Whole plant	12
18	<i>Ocimum sanctum</i>	Full plant	24
19	<i>Pinus roxburghii</i> Sargent	Oleoresin	18
20	<i>Pistacia integerrima</i>	Galls	18
21	<i>Psoralea corylifolia</i> L.	Seeds	18
22	<i>Rhazya stricta</i> Dcne	Leaves	12
23	<i>Rubia cordifolia</i>	Stems	8
24	<i>Sapindus mukorossi</i> Gaertn.	Fruits	24
25	<i>Stenolobium stans</i> (L) D. Don	Roots	18
26	<i>Terminalia arjuna</i> Wight and Arn	Bark	18, 24
27	<i>Trichodesma indicum</i> (L.) R.Br.	Whole plant	18
28	<i>Zingiber officinalis</i> Roscoe	Rhizome	25

Table 2. Effect of various concentration of venom on the amount of free fatty acids released in terms of change in pH of egg yolk suspension.

No.	Concentration of venom (mg/0.1 mL)	Change in pH (mean \pm SEM)	Fatty acid released/min (μ mole)	Enzyme activity (units/mg of crude venom)
1.	Control (saline)	(8 \pm 0)	0	0
2.	0.1	(7.8 \pm 0.028)	26.6	133
3.	0.3	(7.6 \pm 0.028)	53.2	266
4.	0.5	(7.5 \pm 0.028)	66.5	332
5.	1.0	(7.4 \pm 0.028)	79.8	399
6.	2.0	(7.2 \pm 0)	106.4	532
7.	4.0	(7.1 \pm 0.028)	119.7	598
8.	8.0	(7.0 \pm 0.028)	133	665

listed in Table 1 in comparison with reference standard (anti-sera) used in hospitals to treat snake bite.

MATERIALS AND METHODS

Collection of snakes

Naja naja karachiensis (*Naja N. karachiensis*) were collected with the help of local charmers from Cholistan desert located in southern Punjab province of Pakistan. After collection they were dully identified by zoologist.

Snakes venom extraction

Venom from *Naja N. karachiensis* was extracted by compressing the glands below their eyes in low light atmosphere. After collection, it was lyophilized and preserved for further use in light resistant bottle in a refrigerator. Before use it was reconstituted in 0.9% saline in terms of its dry weight (13).

Plants collection

Folklore claimed twenty eight medicinal plants of Pakistan as anti snake venom (Table 1) were collected from various locations in Pakistan. After their collection, they were authenticated by renowned botanist Prof. Dr. Altaf Ahmad Dasti, Bhauddin-Zakariya University, Multan, Pakistan. Voucher specimens were deposited in the herbarium of the Botany department.

Preparation of plants extract

After washing and shade drying different plants material (1 kg) was crushed and subjected to simple maceration process. Methanol (5 L) was used as solvent for extraction and kept in extraction bottles for a period of 4 weeks. They were filtered ini-

tially by muslin cloth followed by Whatman filter paper number 41. Various plants extracts were weighed and preserved for further use after evaporation of methanol in a water bath (14).

Reference standard antidote (anti-venom)

Reference standard antidote (immunoglobulins) was purchased from local pharmacy of Nishtar Hospital, Multan, Pakistan. It was used to compare results of various plants extracts with standard anti-venom. It was manufactured by Bharat Serums and Vaccines Ltd., Ambarnath (E) – 421 501, India (13).

Acidimetric enzymatic assay for snake venom phospholipases A₂

Acidimetric assay for PLA₂ enzymes was followed as described by Tan and Tan (15). Constant volumes of substrate comprising calcium chloride (18 mM), sodium deoxycholate (8.1 mM) and egg yolk were mixed and stirred for 10 min to get homogenous egg yolk suspension. By addition of sodium hydroxide (1 M) pH of the suspension was adjusted to 8.0. Snake venom (0.1–8 mg/0.1 mL) was added to the above mixture (15 mL) to initiate the process of hydrolysis and saline was used as control. A decrease in pH of the suspension was noted after two minutes with the help of a pH meter. A decline in 1.0 pH unit corresponds to the 133 μ moles of fatty acid released in the egg yolk mixture. Furthermore, enzymatic activity of phospholipases A₂ was calculated from the data obtained as micromoles of fatty acid released / minute (15).

To test anti-venom potentials of medicinal plants snake venom (0.1 mg) was pre-incubated with their extracts (0.1–0.6 mg/mL) to neutralize PLA₂ hydrolytic action. Protection offered by vari-

ous plants against phospholipases was measured and represented in terms of percentage.

RESULTS AND DISCUSSION

PLA₂ are the most significant component of cobra venom. In this acidimetric assay, they were hydrolyzed and released free fatty acids in the presence of sodium deoxycholate.

Snake venom was found to liberate free fatty acids in dose dependent manner. This was measured in terms of a decrease in pH of the egg yolk mixture. Snake venom at concentration of 0.1 mg was found to liberate 26.6 μ mol of free fatty acids. However, on increasing the quantity of venom (0.3, 0.5, 1.0, 2.0 and 4.0 mg) large amount of fatty acids (53.2, 66.5, 79.8, 106.4 and 119.7 μ mol/min) were liberated, respectively. Complete drop of 1.0 pH from 8 to

Table 3. Medicinal plants of Pakistan having antidotal properties against *Naja naja karachiensis* phospholipases A₂ in terms of an increase in pH of an egg yolk suspension.

Scientific names of plants	pH at various concentrations of plants extract (mg/mL)			Maximum protection (%)
	0.1	0.3	0.6	
<i>Albizia lebbbeck</i> (L.) Benth.	7.9	7.9	7.9	50
<i>Allium cepa</i> L.	7.9	7.9	7.9	50
<i>Allium sativum</i> L.	7.9	7.9	7.9	50
<i>Althaea officinalis</i> L.	7.9	8.0	8.0	100
<i>Bauhinia variegata</i> L.	8.0	8.0	8.0	100
<i>Brassica nigra</i> (L. Koch)	7.9	7.9	7.9	50
<i>Calotropis procera</i> (Wild.) R.Br. (exudates)	7.9	7.9	7.9	50
<i>Calotropis procera</i> (Wild.) R.Br. (flower)	7.9	7.9	7.9	50
<i>Cedrus deodara</i> G. Don	7.9	7.9	7.9	50
<i>Citrullus colocynthis</i>	7.9	7.9	8.0	100
<i>Citrus limon</i> (L). Burm. f	8.0	8.0	8.0	100
<i>Cuminum cyminum</i> L.	7.9	7.9	8.0	100
<i>Enicostemma hyssopifolium</i> (Willd.) Verdoorn	8.0	8.0	8.0	100
<i>Fogonia cretica</i> L.	7.9	7.9	7.9	50
<i>Leucas capitata</i> Desf.	7.9	7.9	7.9	50
<i>Matthioloa incana</i> (L) R.Br.	8.0	7.9	7.9	100
<i>Momordica charantia</i> L.	7.8	7.8	8	100
<i>Nerium indicum</i> Mill.	7.9	8.0	8.0	100
<i>Ocimum sanctum</i>	8.0	8.0	8.0	100
<i>Pinus roxburghii</i> Sargent	7.9	8.0	8.0	100
<i>Pistacia integerrima</i>	8.0	7.9	7.9	100
<i>Psoralea corylifolia</i> L.	8.0	8.0	8.0	100
<i>Rhazya stricta</i> Dcne	7.9	7.9	7.9	50
<i>Rubia cordifolia</i>	7.9	7.9	7.9	50
<i>Sapindus mukorossi</i> Gaertn.	7.9	7.9	8.0	100
<i>Stenolobium stans</i> (L) D. Don	8.0	8.0	8.0	100
<i>Terminalia arjuna</i> Wight & Arn	7.9	7.9	8.0	100
<i>Trichodesma indicum</i> (L.) R.Br.	7.9	7.9	7.9	50
<i>Zingiber officinalis</i> Roscoe (A-rak)	8.0	7.9	7.9	100
Standard anti-sera (reference standard)	7.9	7.9	7.9	50
Saline	8.0	8.0	8.0	Control

7 (corresponds to 133 μmol of fatty acids) was observed at concentration of 8.0 mg of *Naja naja karachiensis* venom. Enzymatic activity of PLA₂ (units/mg) was found to be elevated as the quantity of venom was improved. Values of 133, 266, 332, 399, 532, 598 and 665 units/mg of PLA₂ were found in 0.1, 0.3, 0.5, 1.0, 2.0, 4.0 and 8.0 mg of crude venom, respectively. Complete data about enzymatic activity of PLA₂ are shown in Table 2.

Twenty eight medicinal plants having concentration ranging from 0.1 to 0.6 mg/mL were evaluated to neutralize venom's phospholipases activity. All medicinal plants were found to have potentials to inhibit phospholipases hydrolytic action but vary in their potency. Among them *Bauhinia variegata* L., *Citrus limon* (L.) Burm. f., *Enicostemma hyssopifolium* (Willd.) Verdoorn, *Ocimum sanctum*, *Psoralea corylifolia* L. and *Stenobolium stans* (L.) D. Don were effective at 0.1 mg/mL to inhibit complete PLA₂ hydrolytic action(s). Their lower doses were equally valuable as their higher doses to bestow 100% protection. *Althaea officinalis* L., *Citrullus colocynthis*, *Cuminum cyminum* L., *Momordica charantia* L., *Nerium indicum* Mill., *Pinus roxburghii* Sargent, *Sapindus mukorossi* Gaertn., *Terminalia arjuna* Wight & Arn showed their potentials at higher concentration (0.3 or 0.6 mg/mL) to inhibit completely (100%) PLA₂ enzymatic actions. Three plants (*Matthiola incana* (L.) R.Br., *Pistacia integerima* and *Zingiber officinalis* Roscoe) were recorded to halt 100% PLA₂ activity only at lower dose (0.1 mg/mL). Their higher concentrations were proved to aggravate venom response. Rest of all plants was found to neutralize 50% PLA₂ hydrolytic actions at all concentrations (0.1–0.6 mg/mL). Among them *Albizia lebeck* (L.) Benth., *Allium cepa* L., *Allium sativum* L., *Brassica nigra* (L. Koch), *Calotropis procera* Wild. (flowers and exudates), *Cedrus deodara* G. Don, *Fogonia cretica* L., *Leucas capitata* Desf., *Rhazya stricta* Dcne, *Rubia cordifolia* and *Trichodesma indicum* (L.) R.Br. are included. Standard antidote was found 50% effective to combat poisonous effects of PLA₂. Complete detail about working efficiencies of various antidotes have been summarized in Table 3.

People living in tropical and subtropical areas of the world are awfully more prone to snake bite envenomation. Among various noxious proteins of cobra, venom PLA₂ are in abundance and play a pivotal role in emergence of toxicities. They are tiny richest molecules that cause havoc by alteration in physiological processes within victims (16).

PLA₂ were found to interact with other components particularly lipids of the cells. Both specific

and non specific PLA₂ developed protein – phospholipids interaction with covalent, non covalent or disulfide interactions. PLA₂ (Asp49 variants) caused to hydrolyze phospholipids or released free fatty acids (16, 17). This was the reason when quantity of snake venom was increased it liberated greater amount of free fatty acids (in the presence of deoxycholic acid) due to abundance of PLA₂.

There are numerous protein (enzyme) binding constituents that have been reported previously to neutralize snake venom peptides. Medicinal plants are stuffed with these compounds, therefore have engrossed therapeutic priorities in application of natural inhibitors. Medicinal plants of Pakistan have primeval record to neutralize snake venom proteins due to abundance of miscellaneous secondary metabolites. Among them quinonoids, terpenoids, polyphenols, xanthenes and flavonoids were documented previously to minimize snake venom toxins (12).

CONCLUSION

On above grounds, present research work has confirmed folklore claims (as anti-snake venom) about medicinal plants to rationalize them scientifically in traditional system of medicine. Among 28 medicinal plants extracts only six were found to provide 100% protection against PLA₂ hydrolytic actions at minimum concentration of 0.1 $\mu\text{g/mL}$. They were proved more potent and effective when compared with standard antidote (reference standard). However, further study is inevitable for discovery and isolation of bioactive constituent(s) from six the most potent and effective medicinal plants to handle adroitly the problem of snake poisoning.

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ANTIOXIDANT AND HYPOGLYCEMIC EFFECT OF *OTOSTEGIA AUCHERI* METHANOLIC EXTRACT IN STREPTOZOTOCIN-INDUCED DIABETIC MALE LONG-EVANS RATS

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Abstract: Present study is based on the investigation of antioxidant and antihyperglycemic effect of methanolic extract from areal parts of *Otostegia aucheri* (OA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) method was used to measure the antioxidant activity of extract of the species *Otostegia aucheri*. The observed scavenging activity for the free radicals was significant and it was compared with the standard BHT inhibition method. The IC₅₀ value obtained of methanolic extract was 2.23 µg/mL. The methanolic extract of OA on the blood glucose level was further studied in normal (non-diabetic), streptozotocin (STZ)-induced type I and type II diabetic male Long-Evans rats at postprandial glucose load state. The results revealed that the oral administration of methanolic extract (1.25 g/kg) of OA showed no remarkable hypoglycemic effect in normal and type I (IDDM) diabetic rats. However, the methanolic extract significantly lowered ($p < 0.005$) serum glucose level in type II diabetic (NIDDM) models when simultaneous glucose was administered. This screening for antioxidant activity interprets the pernicious effects of diabetes that have been associated with mediation through the oxidation stress. The study also suggests to introduce natural source of the potential orally active antioxidant and active antihyperglycemic phytochemicals for the future. It may also improve the impaired antioxidant defense system.

Keywords: *Otostegia aucheri* L., antioxidant activity, hypoglycemic effect, streptozotocin, diabetes

The medicinal plants contribute a lot in support of human communities all over the world (1). In addition to other uses, plants remained the main source of medicines. The traditional plant remedies have always been pragmatically practiced for various ailments like diabetes, hypertension, jaundice, cardiovascular diseases and cancer in traditional ways (2). An endocrine disorderness is the major cause of diabetes mellitus (DM) and oral hypoglycemic drugs and/or insulin is used generally for the treatment (3). As the immune system of the body diminishes, the pancreatic β cells that produce insulin for the regulation of blood glucose level, get affected, hence, develop type I (IDDM) diabetes. In type II (NIDDM) diabetes, resistance is developed against the use of insulin by cells. As a result, the need for insulin increases and pancreas gradually loses its ability to produce insulin. Although pathogenesis of both IDDM and NIDDM diabetes are varied, hyperglycemia and its related complications are common to some extent in both types (4). Cardiovascular complications are due to the

increased level of lipid profile in the diabetes (5). The production of free radicals is usually increased in diabetes, hence, simultaneous decline of antioxidants defense mechanisms (6) causes oxidative stress (7). The elevation of oxidative stress consequently prop up the complications (8).

In fact, diabetes is manageable using pharmacologic products and changing life style, like controlled diet and light exercise (9, 10) along with taking effective antidiabetes phyto-medicines. Since all of the pharmacologic agents are exceptionally not without severe side effects, so the researchers are motivated to seek for remedies in traditional medicines that have milder toxicity than available allopathic drugs (11). Diabetes complications have lethal effects due to multiple defects in its pathophysiology (12). Current research trend is diverted towards traditional medicinal herbs and plants as potential alternative source to manage diabetes with its multiple pharmacologic actions (13). A large number of antidiabetic phytoconstituents were isolated and characterized from natural sources and still

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research is continuously focused to explore new antidiabetic better natural lead molecules for the benefit of humanity (14).

Although the complementary and alternative medicine consists of phytomedicine and nutraceuticals as alternatives, to mainstream allopathic treatment is another better option. According to the recent estimation, up to 30% diabetics take dietary supplements as alternative medicine (15). In the third world countries hypoglycemic plants contribute a lot in controlling and management of diabetes (16). Natural products from various sources like plants, animals and microorganisms are considered to be strong candidate of pharmaceutical drugs. It has been clearly described in the ethnobotanical literature that a large number of plants species with antidiabetic potential are used worldwide (17).

In Balochistan, there is a wide arsenal of medicinal plants because of the high altitude. A huge number of medicinal plants with antidiabetic efficacy are yet to be scientifically studied and commercially formulated as modern drugs, since they have been commended for their potential hypoglycemic therapeutic in traditional medicine (18).

The genus *Otostegia* belongs to family Lamiaceae and consists of more than 31 species growing in Asia and Mediterranean region (19).

Only three species are known in Pakistan, *Otostegia limbata*, *Otostegia persica* and *Otostegia aucheri* (20). They are all biologically active and were subjected to the phytochemical isolation and characterization studies. Alcoholic extract of *O. persica* showed hypoglycemic and hypolipidemic effect in normoglycemic and streptozotocin-induced diabetic rats with significant antioxidant efficacy (21).

In recent research, indigenous plant *O. aucheri* was subjected to evaluation of the antioxidant and anti-diabetes efficacy on account of the reported literature (22).

The traditional medicinal plants should be investigated to get better understanding of their chemistry to elucidate the structure of the bioactive constituents, their efficacy, selectivity, specificity and safety. Thus, the provision of new aspects may solve the health and economical challenges in the region (23).

In the presented studies, methanolic extract of indigenous plant species *Otostegia aucheri* (OA) have been taken into consideration to investigate the antioxidant potential using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity assay. The pharmacological screening of the plant extract revealed potent antioxidant activity. Further

in vivo antidiabetic activity for the crude plant extract was also determined by streptozotocin (STZ)-induced diabetic model in rats. These results add new imminence for exploitation of source of natural antioxidant and antidiabetic drugs as the screened plant exemplified potential. Further research is awaited to explore and identify the bioactive moieties from the fractions present in the crude extract and also to determine their full spectrum of efficacy, selectivity and specificity.

EXPERIMENTAL

Plant material

The plant *Otostegia aucheri* (Lamiaceae) local name Gul Gaider was collected from Kohlu, Balochistan province, Pakistan. The species was confirmed by Prof. G. Rasool Tareen (Department of Botany, University of Balochistan, Quetta, Pakistan) and specimens (Voucher no. 335) were deposited in the herbarium of Department of Botany, University of Balochistan, Quetta, Pakistan.

Extraction

The air dried aerial parts of *O. aucheri* were finely powdered, and 10 kg sample was soaked in 80% methanol for a week. It was exhaustively extracted with methanol (3 × 10 L). The extract was evaporated under reduced pressure to yield the residue, 1.52 kg.

In vitro antioxidant assay

In this experiment, methanolic extract of *O. aucheri* and standard BHT ((butylated hydroxytoluene) were subjected to evaluation of antioxidant activity. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was used for measuring free radical scavenging activity (RSA) IC₅₀ (24). *O. aucheri* extract (90, 60, 30, 3.0 µg/mL) in methanol were added to 2 mL of methanolic DPPH solution (4 × 10⁻⁵ g/mL MeOH). The BHT standard was used as control. The absorption of sample was measured at 517 nm after 30 min. The percentage of inhibition of activity was calculated as follows:

$$\text{Inhibition (\%)} = 100 - \frac{(\text{Sample Absorption} - \text{Control Absorption})}{\text{Blank Absorption}} \times 100$$

Analyses of at least 3 samples were carried out in triplicate.

Antidiabetic bioassay

Induction of diabetes

Male Long-Evans rats (150–210 g) were used to screen the efficacy of the crude extract. Only single dose administration of the crude extract with

simultaneous glucose was fed to normal rats, insulin dependent diabetes mellitus (IDDM) models and noninsulin dependent diabetes mellitus (NIDDM) rats.

The standard conditions of temperature and relative humidity, with a 12 h light/dark cycle were maintained in animal house. Water and commercial rat feed were provided *ad libitum*. The current experiments were carried out with a prior permission from the institutional ethical committee regarding animals used in experiments. The IDDM diabetes was induced in rats by single interperitoneal injection of freshly prepared streptozotocin (STZ) solution in citrate buffer (pH 4.5) to 12 h fasted adult rats at a dose of 65 mg/kg body weight. After 84 h of STZ administration, the serum glucose level of each rat was determined for confirmation of diabetes.

Type II diabetes (NIDDM) was produced by single intraperitoneal injection of freshly prepared streptozotocin (STZ) solution to 48 h old pups at a dose of 90 mg/kg body weight (25). The diabetic status of the models was checked prior to experimentation by blood glucose estimation. Rats with serum glucose level above 250 mg/dL were considered as diabetic and were used in the experiments.

Plant extract administration with simultaneous glucose load

The rats were fasting for 12 h, but they had free access to water. The alcoholic extract of *O. aucheri* (1.25 g/kg body weight in 2 mL distilled water) suspension was orally administered simultaneously with glucose (2.5 g/kg body weight) by gastric tube attached with 5 mL syringe. The experimental control groups received only glucose at the same dose.

Blood sample collection

Keeping experimental rats under mild ether anesthesia, blood samples were collected by cutting the tail tip, after immersing tail in luke-warm water for 30 s for vascular dilation. The samples were collected at zero minute followed by 30 and 75 min intervals. The serum was separated by centrifuga-

tion at the rate of 5700 rpm for 5 min. The glucose level was measured immediately and rest of serum was stored at -20°C until further analyzed.

Estimation of blood glucose level

The glucose level of serum was immediately estimated by enzymatic-colorimetric method using glucose-oxidase method GOD-PAP (26). The absorbance was measured by a microplate reader (Bio-Tek, ELISA). The 10 mL of 20 mmol glucose solution was prepared in different dilutions 8, 6, 4, 2 and 1 mmol/L and distilled water was used as blank with zero mmol/L glucose. Serum sample (2.5 μL) with 5 μL of each concentration of the glucose solution were dispensed into the wells of the micro titer plate and on top of that 200 μL glucose oxidase GOD-PAP was added simultaneously by multi-channel pipette, incubated for 15 min at 37°C and the absorbance was measured at 490 nm using microplate ELISA reader (Bio-Tek EL.). Each value represents the mean of duplicate measurements.

Statistical analysis

All results are tabulated as the mean \pm SEM. Data analysis was done by applying unpaired Student's *t*-test and results were compared to mean values between control and treated groups. Values of *p* less than 0.05 and 0.005 in different experiments were considered significant.

RESULTS AND DISCUSSION

The antioxidant and antidiabetic activities of *O. aucheri* (AO) are presented in the tabular form. The statistical analysis of the antioxidant activity showed significant ($p < 0.05$) differences between results of extract and BHT, the results indicated that the methanolic extract has remarkable free radical scavenging activity compared to standard (Table 1).

IC₅₀ of samples from *O. aucheri* with DPPH method

In male rats, it has been reported that oral single administration of extract significantly lower the

Table 1. IC₅₀ of samples from *O. aucheri* with DPPH method.

Samples/Standard	DPPH/30 min ($\mu\text{g/mL}$)
<i>Otostegia aucheri</i> extract	2.23 \pm 0.97 *
BHT (Butylated hydroxytoluene)	110.97 \pm 8.25

* Values are the mean \pm SD (n = 3), $p < 0.05$ considered significant

Table 2. Effect of *O. aucheri* extract on the serum glucose level (mmol/L) in normal (non diabetic), IDDM and NIDDM model rats.

Treatment groups	0 min	30 min	75 min	Δ
Normal (non-diabetic) rats				
Control (n = 12)	8.28 ± 0.22	11.94 ± 0.42	9.5 ± 0.30	4.92 ± 0.87
<i>O. aucheri</i> (n = 9)	7.74 ± 0.23	11.9 ± 0.51	10.0 ± 0.45	6.50 ± 0.58
IDDM model rats				
Control (n = 8)	24.3 ± 1.6	31.60 ± 1.89	27.4 ± 1.7	10.3 ± 2.9
<i>O. aucheri</i> (n = 8)	22.9 ± 1.32	29.9 ± 1.2	24.3 ± 1.03	8.3 ± 2.8
NIDDM model rats				
Control (n = 10)	8.8 ± 0.39	20.1 ± 1.76	15.7 ± 2.1	18.2 ± 3.2
<i>O. aucheri</i> (n = 7)	9.4 ± 0.50	11.3 ± 1.3*	10.9 ± 1.6	3.23 ± 2.5

Data are expressed in terms of means ± SEM; 'n' indicates the number of rats in each groups. Δ, cumulative increment over basal value, * significant compared to baseline value (0 minute)

serum glucose level and increase insulin level. Even biologically active phytochemicals are potential source of several modern drugs, which produce hypoglycemia in the diabetic animals and non-significant in normal animals (27).

The oxidative stress mediated deadly effects of diabetes, since it is associated with increased release of reactive oxygen species and impaired antioxidant immune system. Due to this lipid peroxidation, change in antioxidants enzymes, impaired glutathione metabolism and a decrease in ascorbic acid occurred mainly on account of ineffective scavenging of reactive oxygen species that play a pronounced role in diabetes mellitus complications. Hence, disturbance of antioxidant defense system in diabetes mellitus (28, 29). The alcoholic extract was further analyzed for its hypoglycemic activity.

As shown by the results, there was not remarkable change in the normal rats and IDDM model upon feeding the methanolic extract of *Ostostigea aucheri* with simultaneous glucose load (Table 2). However, the oral administration of methanolic extract of *O. aucheri* significantly lowered the blood glucose level in NIDDM rats. The extract revealed to be proficient in lowering sugar levels within 30 min (*p < 0.005), thus indicating that the extract of *O. aucheri* might not affect insulin secretion by pancreatic cells in NIDDM rats. It may delay the development of the diabetic complications. The plant extract of *O. aucheri* may be adapting the extra-pancreatic mechanism as antidiabetes agent, which resembles the mode of action of biguanides drugs (30).

As the isolated phytoconstituents of this plant have not been studied and characterized as antidiabetic agents, therefore, the mechanism of mode of

action of the extract as antidiabetic and antioxidant is focused. This study is in sequence to an ethnobotanical assessment of medicinal plant alleged for the cure of diabetes mellitus. Comparative available literature study with other ethnobotanical analysis of plants used conventionally for the management of diabetes mellitus recommends that plant genus build claim of new information on antidiabetic usefulness. According to literature, antidiabetic drugs increase the blood insulin levels of diabetic rats by improving the antioxidant status and decreased lipid peroxidation (31).

Present results support the use of *O. aucheri* as an antidiabetic agent by conventional healers in urban environment. Hence, it is necessary to open new path for developing broad-spectrum antidiabetic drugs. The administration of new plant used in the present research showed the significance of the wide variety of such ethnobotanical understanding. The executive use of *O. aucheri* results in considerable restoration of the blood glucose levels.

CONCLUSION

O. aucheri exhibited powerful antioxidant and hypoglycemic effect in type II diabetic model rats. It is known that oxidative stress is produced under diabetic conditions and *O. aucheri* extract is considered for significant hypoglycemic activity as it is also a good antioxidant. It can be utilized as natural antioxidant and a preventing agent for diseases caused by free radicals and as antidiabetic in terms of associated complications. However, further investigations must be conducted to evaluate the mode of action with antidiabetic and antioxidant effect of the plant extract and identification of phytochemicals respon-

sible for this action. These activities may not only be attributed to the presence of potential antioxidant and antidiabetic molecule of the future but also provide appropriate mechanism to explore the interconnected management of diabetes complications through natural antioxidant.

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PHARMACEUTICAL TECHNOLOGY

USEFULNESS OF ROSEN'S POSTULATE FOR STUDYING
THE RELATIONSHIP BETWEEN THE STRUCTURE OF CHOLIC ACID
OXYETHYLATION PRODUCTS AND THE PROCESS OF SOLUBILIZATION
OF LIPOPHILIC THERAPEUTIC AGENTS (BCS CLASS II AND IV)
IN AQUEOUS SOLUTIONS IN EQUILIBRIUM

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Abstract: We verified the usefulness of "Rosen's postulate", i.e., the logarithm of reciprocal concentration of surfactant $-\log(1/c_{n=20})$ by which the surface tension of a solution can be decreased by 20 mJ/m² in relation to water (physiological value $\gamma^s = 48-52$ mJ/m²) in the evaluation of the applicatory properties of cholic acid oxyethylation products. Moreover, the values of ΔG_m^0 for solubilizers and their micellar adducts with diclofenac, naproxen, and loratadine constituted the basis for estimating the thermodynamic value of "Rebinder's effect", associated with change in the state of matter of therapeutic agent. We determined critical micellar concentration for the aqueous products of oxyethylation and for micellar adducts with diclofenac, naproxen, and loratadine, and used these values to calculate (thermodynamic potential of micelle formation).

Keywords: oxyethylation, cholic acid, oxyethylates of cholic acid, surfactants, Rosen's postulate

Rosen is known for implementing p value to the analysis of the surface activity of surfactants. This value is the logarithm of reciprocal concentration by which the surface tension of water can be decreased by 20 mN/m (1). Revealing the linear relationship between $\log(1/c_{n=20})$ and the number of carbon atoms in the lipophilic radical $[(n_c)]$ of the homological structures of ionic and non-ionic surfactants constitutes the basis for using the directional coefficients of correlation equation to calculate the energy of transport to the phase boundary of lipophilic radical $-\Delta G_{tr(1)}[F_w \uparrow]$, as well as to the hydrophilic part of surfactant molecule $-\Delta G_{tr(h)}[F_c \downarrow]$.

Previous application studies of the surface activity and solubilization properties of the aqueous solutions of Pluronics and Rokopols (2), M-PEG (3, 4), and Rokanols and Rokacets (5, 6) constituted the basis for calculating $\log(1/c_{n=20})$ value and analyzing its relationship with the number of oxyethylated segments (CH_2CH_2O) in surfactant structure (7). The calculated values of transport

energy, $\Delta G_{tr(h)}$ for hydrophilic segments $(CH_2CH_2O)_{n=1}$ and $\Delta G_{tr(l)}$ for lipophilic radicals allowed, in response to Laughlin's postulate (8), to propose H/L $\Delta G_{tr}(h/l)$ ratio as the thermodynamic version of hydrophilic-lipophilic balance (H/L, HLB). The previously published (9) thermodynamic approach to the hydrophilic-lipophilic balance H/L (ΔG_{tr}) of surfactants can explain the process of the micellar solubilization of lipophilic therapeutic agent (solid body) in equilibrium by the micellar solution of surfactant ($c_{exp} \geq c_{mc}$) at phase boundary. These findings constituted the basis for verifying the possibility of using "Rosen's postulate" not only to determine the surface activity of the aqueous solutions of cholic acid oxyethylation products with $n_{TE} = 20-70$, but also to explain the so-called Rebinder's effect, which in thermodynamic approach accompanies the process of the solubilization of lipophilic therapeutic agents (BCS class II and IV) in equilibrium in the aqueous solutions of surfactant with $c_{exp} \geq c_{mc}$ (critical micellar concentration) (10–14).

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Table 1. Correlation formulas describing relationship: $\gamma_{\text{res}}^{\text{res}} = f(c, \text{mol}/\text{dm}^3)$, constituting the basis for calculating "Rosen's postulate" – $\log(1/c_{\text{res}}^{20})$ – along with the determined number of n_{TE} and $\text{HLB}^{\text{UNIBR}}$ for solubilizer and its micellar adducts with diclofenac, naproxen, and loratadine.

Solubilizer Cholic acid Therapeutic agent	Determined n_{TE}	$\text{HLB}^{\text{UNIBR}}$	$\Delta C_{\text{m}}^{\text{m}}$ kJ/mol	Correlation formula: $\gamma_{\text{res}}^{\text{res}} = f(c, \text{mol}/\text{dm}^3) \cdot c_{\text{res}}^{20}$			$\times 10^4 \text{ mol}/\text{dm}^3$	$\log[1/c_{\text{res}}^{20}]$
				r^2	$a \pm \text{da}[\gamma_{\text{res}}^{\text{res}}]$	$b \pm \text{db}$		
1. Cholic acid $n_{\text{TE}} = 20$	23.79	12.85	-19.7647	0.9708	69.7142 ± 3.6484	-175235.2 ± 79491.3	1.0125	3.9945
2. Cholic acid $n_{\text{TE}} = 30$	29.88	13.14	-19.6345	0.9901	70.3569 ± 1.2116	-179686.4 ± 47059.3	1.0232	3.9900
3. Cholic acid $n_{\text{TE}} = 40$	42.66	15.01	-19.8823	0.9918	71.2818 ± 0.9700	-180235.1 ± 42488.9	1.0714	3.9700
4. Cholic acid $n_{\text{TE}} = 50$	50.11	16.59	-20.2497	0.9755	70.7455 ± 1.8763	-242231.4 ± 100258.7	1.1342	3.9453
5. Cholic acid $n_{\text{TE}} = 60$	57.28	17.79	-20.5486	0.9761	69.5929 ± 2.1544	-149315.4 ± 61096.9	1.1802	3.9280
6. Cholic acid $n_{\text{TE}} = 70$	73.57	19.22	-21.8211	0.9803	66.4608 ± 1.0527	-110624.0 ± 40920.2	1.3099	3.8828
Diclofenac – adduct								
1. Cholic acid $n_{\text{TE}} = 20$	20.37	15.59	-19.4925	0.9921	69.8262 ± 1.1407	-194765.3 ± 45202.7	0.9168	4.0377
2. Cholic acid $n_{\text{TE}} = 30$	28.01	16.54	-19.5131	0.9901	70.5182 ± 1.4522	-227375.0 ± 59239.5	0.8157	4.0884
3. Cholic acid $n_{\text{TE}} = 40$	39.11	17.38	-20.2899	0.9919	69.8224 ± 1.1555	-223134.0 ± 52425.8	0.8001	4.0968
4. Cholic acid $n_{\text{TE}} = 50$	51.85	17.95	-20.7147	0.9808	68.7459 ± 1.4252	-244641.0 ± 89154.6	0.6857	4.1638
5. Cholic acid $n_{\text{TE}} = 60$	57.22	18.12	-21.0969	0.9523	69.1569 ± 2.4991	-332979.1 ± 195952.1	0.5161	4.2872
6. Cholic acid $n_{\text{TE}} = 70$	67.09	18.37	-21.7467	0.9779	70.7762 ± 1.9292	-395400.7 ± 155289.6	0.4756	4.3272
Naproxen – adduct								
1. Cholic acid $n_{\text{TE}} = 20$	19.22	16.66	-19.7322	0.9842	71.0299 ± 1.7985	-238866.8 ± 78829.1	0.7979	4.0980
2. Cholic acid $n_{\text{TE}} = 30$	30.09	16.74	-19.7898	0.9919	69.8225 ± 1.1555	-223134.0 ± 52425.8	0.8001	4.0968
3. Cholic acid $n_{\text{TE}} = 40$	40.77	17.44	-20.1698	0.9811	69.3128 ± 1.4891	-221784.4 ± 78742.7	0.7961	4.0990
4. Cholic acid $n_{\text{TE}} = 50$	51.01	17.64	-20.7838	0.9565	70.3319 ± 2.6044	-314868.7 ± 176413.8	0.5831	4.2342
5. Cholic acid $n_{\text{TE}} = 60$	56.61	18.11	-20.9758	0.9921	69.5545 ± 1.1025	-347707.3 ± 80695.2	0.5057	4.2961
6. Cholic acid $n_{\text{TE}} = 70$	65.29	18.33	-21.5748	0.9374	67.2716 ± 2.6267	-347344.5 ± 237046.9	0.4405	4.3560
Loratadine – adduct								
1. Cholic acid $n_{\text{TE}} = 20$	22.21	15.85	-19.6127	0.9803	66.4607 ± 1.0528	-110624.0 ± 40920.2	1.3099	3.8227
2. Cholic acid $n_{\text{TE}} = 30$	31.39	16.85	-19.2241	0.9991	70.1485 ± 0.3937	-176915.3 ± 13117.5	1.0275	3.9882
3. Cholic acid $n_{\text{TE}} = 40$	42.98	17.58	-20.6948	0.9792	70.7474 ± 2.3500	-261099.0 ± 99251.3	0.7191	4.1431
4. Cholic acid $n_{\text{TE}} = 50$	51.99	17.64	-20.9836	0.9565	70.3319 ± 2.6044	-314868.0 ± 80695.2	0.5831	4.2348
5. Cholic acid $n_{\text{TE}} = 60$	56.28	18.09	-20.5157	0.9547	64.9535 ± 1.9643	-336857.9 ± 192723.6	0.3854	4.4140
6. Cholic acid $n_{\text{TE}} = 70$	69.81	18.42	-21.9692	0.9927	65.4909 ± 0.7084	-943116.9 ± 209107.7	0.1433	4.38435

Table 2. Correlation formulas describing "Rosen's postulate", and the energy required to transport the oxyethylated segment $[\text{CH}_2\text{CH}_2\text{O}]_{n_{\text{TE}}}$ and lipophilic segment (adduct with therapeutic agent) from solution to interphasic interface: aqueous solution – air.

Solubilizer Cholic acid n_{TE} Therapeutic agent	Type of correlation formula	Directional coefficients of the formulas		$\Delta G_{\text{TE}}[\text{CH}_2\text{CH}_2\text{O}]_{n_{\text{TE}}}(\text{h})$ [KJ/mol]	$\Delta G_{\text{TE}}[\text{R-}]$ (l) lipophilic segment [KJ/mol]
		r^2	$a \pm da$		
1. Cholic acid $n_{\text{TE}} = 20-70$	$y = a + b \times n_{\text{TE}}$	0.9879	4.0576 ± 0.0251	$-2.2905 \times 10^{-3} \pm 0.5109 \times 10^{-3}$	0.401205×10^{-3}
	$\log y = a + b \gamma \times n_{\text{TE}}$	0.9862	0.6084 ± 0.0028	$-2.5231 \times 10^{-4} \pm 0.5743 \times 10^{-4}$	
2. Cholic acid $n_{\text{TE}} = 20-70$ + Diclofenac	$y = a + b \times n_{\text{TE}}$	0.9491	3.0576 ± 0.1334	$-6.1701 \times 10^{-3} \pm 2.8445 \times 10^{-3}$	1.08068×10^{-3}
	$\log y = a + b \gamma \times n_{\text{TE}}$	0.9508	0.5914 ± 0.0136	$6.4118 \times 10^{-4} \pm 2.9028 \times 10^{-4}$	
3. Cholic acid $n_{\text{TE}} = 20-70$ + Naproxen	$y = a + b \times n_{\text{TE}}$	0.9178	3.9281 ± 0.1718	$-6.1240 \times 10^{-3} \pm 3.6904 \times 10^{-3}$	1.072611×10^{-3}
	$1/y = a + b \times n_{\text{TE}}$	0.9178	0.2535 ± 0.0096	$-3.4546 \times 10^{-4} \pm 2.0744 \times 10^{-4}$	
4. Cholic acid $n_{\text{TE}} = 20-70$ + Loratadine	$y = a + b \times n_{\text{TE}}$	0.9594	3.3759 ± 0.3776	$1.9117 \times 10^{-2} \pm 0.7797 \times 10^{-2}$	3.348320×10^{-3}
	$1/y = a + b \times n_{\text{TE}}$	0.9758	0.2836 ± 0.0155	$-1.0331 \times 10^{-3} \pm 0.3205 \times 10^{-3}$	

Table 3. Values of $H/L(\Delta G_{\text{TE}})$ ratio determined for cholic acid oxyethylation products and for micellar adducts with diclofenac, naproxen, and loratadine.

Equilibrium system Cholic acid n_{TE} Therapeutic agent	$H/L(\Delta G_{\text{TE}}) \times 10^3$		
	Solubilizer with diclofenac	Micellar adduct with naproxen	Micellar adduct with loratadine
1. Cholic acid $n_{\text{TE}} = 20$	42.1082	12.9682	12.3281
2. Cholic acid $n_{\text{TE}} = 30$	52.9220	17.6849	19.3005
3. Cholic acid $n_{\text{TE}} = 40$	75.5716	24.6932	26.1509
4. Cholic acid $n_{\text{TE}} = 50$	88.7692	32.7371	32.7192
5. Cholic acid $n_{\text{TE}} = 60$	101.4708	36.1275	36.3110
6. Cholic acid $n_{\text{TE}} = 70$	130.3284	42.3593	41.8786
$H/L(\Delta G_{\text{TE}}) = n_{\text{TE}} \cdot \Delta G_{\text{TE}} / \Delta G_{\text{TE}}(\text{h})$			

Table 4. Correlation formulas describing relationship between G_m^0 and HLB^{1HNMR} for solubilizer and its micellar adducts with therapeutic agents as a function of n_{TE} .

Solubilizer Cholic acid n_{TE} Therapeutic agent	Type of correlation formula for function	Directional coefficients of the formulas		
		r^2	$a \pm da$	$a \pm da$
	$\Delta G_m^0 = f(n_{TE})$			
	$y = a + b \times x$			
1. Cholic acid $n_{TE} = 20-70$	$y = a + b \times x$	0.9262	-18.4190 ± 1.1394	$-4.1065 \times 10^2 \pm 2.3193 \times 10^2$
2. Cholic acid $n_{TE} = 20-70 +$ Diclofenac	$y = a + b \times x$	0.9768	-18.5966 ± 0.5766	$-4.0485 \times 10^2 \pm 1.2297 \times 10^2$
3. Cholic acid $n_{TE} = 20-70 +$ Naproxen	$y = a + b \times x$	0.9691	-18.7013 ± 0.6763	$-4.1135 \times 10^2 \pm 1.4526 \times 10^2$
4. Cholic acid $n_{TE} = 20-70 +$ Loratadine	$y = a + b \times x$	0.9567	-17.6187 ± 2.2685	$-5.6061 \times 10^2 \pm 4.6846 \times 10^2$
	$HLB^{1HNMR} = f(n_{TE})$			
	$y = a + b \times x$			
1. Cholic acid $n_{TE} = 20-70$	$y = a + b \times x$	0.9896	9.3678 ± 1.3698	0.1384 ± 0.0278
2. Cholic acid $n_{TE} = 20-70 +$ Diclofenac	$y = a + b \times x$	0.9644	14.7743 ± 1.0342	0.0581 ± 0.0221
3. Cholic acid $n_{TE} = 20-70 +$ Naproxen	$y = a + b \times x$	0.9788	15.7706 ± 0.5291	0.0391 ± 0.0113
4. Cholic acid $n_{TE} = 20-70 +$ Loratadine	$y = a + b \times x$	0.9609	15.0441 ± 0.9988	0.0515 ± 0.0206

Consequently, the values of cmc determined for the aqueous solutions of the oxyethylation products and micellar adducts with diclofenac, naproxen, and loratadine were used to calculate ΔG_m^0 (thermodynamic potential of micelle formation). Moreover, the total solids obtained after the evaporation of water ($\sim 37^\circ\text{C}$) from saturated micellar solutions formed in the process of solubilization in equilibrium constituted the basis for determining the content of oxyethylated segments, n_{TE} and HLB^{1HNMR} , using $^1\text{HNMR}$ method. The results of these studies, along with the aggregates formed as a result of evaporating water from saturated micellar solutions formed during the micellar solubilization of diclofenac, naproxen, and loratadine in equilibrium, constituted significant material deliverables of the verification of the usefulness of "Rosen's postulate" for the thermodynamic estimation of energy required to change the state of matter at phase boundary: therapeutic agent (solid body) – micellar solution of surfactant with $c_{exp} \geq \text{cmc}$. The aim of the research reported here was to confirm the usefulness of Rosen's proposal for estimating the solubilizing properties and the thermodynamic stability of the micellar adduct.

EXPERIMENTAL

Materials

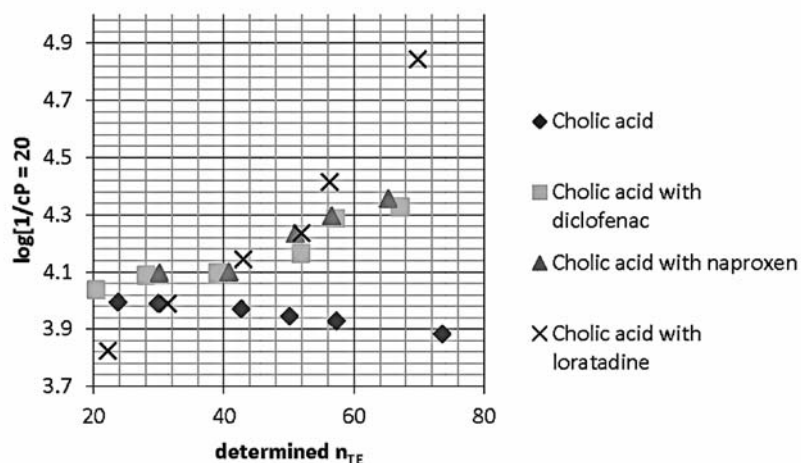
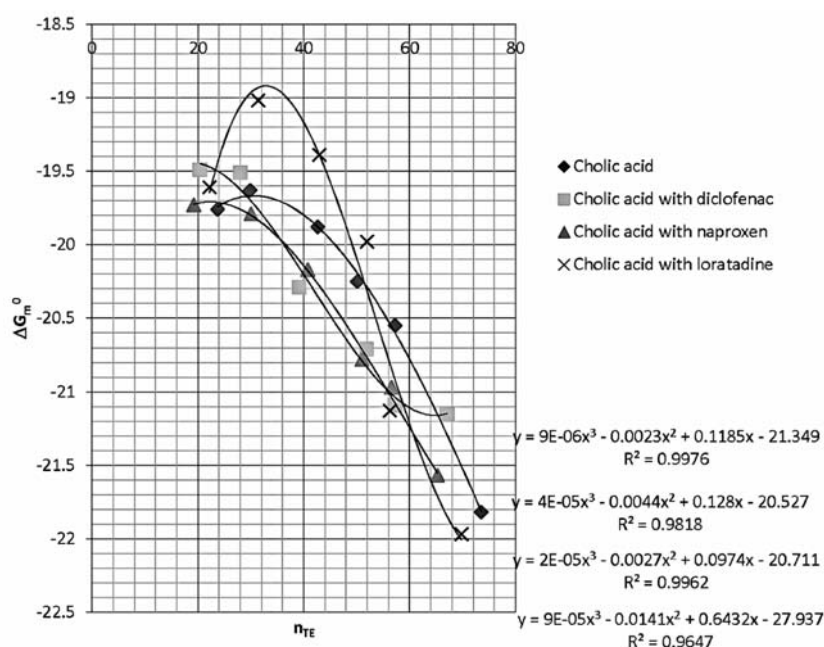
Product of the catalytic oxyethylation of cholic acid with declared molar content of ethylene oxide – $n_{TE} = 20-70$. Structural characteristics and aqueous solutions of the novel class of surfactants were described in our previous publication (10). Diclofenac: 2-{2-[(2,6-dichlorophenyl)amino]phenyl}acetic acid, pure for analysis (Sigma, Germany). Naproxen: 2-(6-methoxy-2-naphthyl)propionic acid, pure for analysis (Zydus Cadila – Cadila Healthcare Ltd., India). Loratadine: 4-(8-chloro-5,6-dihydro-11H-benzo[5,6]cyclohepta[1,2b]pyridin-11-ylidene)-1-piperidine-1-carboxylic acid ethyl ester, pure for analysis (Zydus Cadila – Cadila Healthcare Ltd., India). All the substances used in the course of research have a low level of water solubility and belong to BCS Class II.

Equipment

Stalagmometers with $V = 28.20 \text{ cm}^3$ and $V = 45.0 \text{ cm}^3$ (Tropfen Wasser type) with MLW-U2C type thermostatic system of measurement area (Medingen Sitz Freital, Germany).

Research methodology

The surface activity of aqueous solutions of the products of cholic acid oxyethylation and their

Figure 1. Graphic illustration of relationship: $\log(1/c_{P=20}) = f(n_{TE})$ Figure 2. Graphic illustration of relationship: $DG_m^0 = f(n_{TE})$

adducts formed as a result of the micellar solubilization of diclofenac, naproxen, and loratadine in equilibrium was determined on the basis of changes in surface tension. The surface activity of the aqueous solutions of solubilizers and their micellar adducts with the lipophilic therapeutic agents was determined with the stalagmometric method in accordance with the Polish Standard (15).

Determination of cmc

The determined value of critical micellar concentration (cmc) allowed for the calculation of the thermodynamic potential of micelle formation (Δ) based on the following equation:

$$\Delta G_m^0 = 2.303RT \cdot \log(\text{cmc})$$

The relationship $\gamma^{25} = f(c, \text{mol/dm}^3)$ in the examined range of concentrations, i.e., for the val-

ues below cmc, was described with regression equations at $p = 0.05$. The correlation equations presented in Table 1 allowed us to calculate $c_{\pi=20}$ and $\log(1/c_{\pi=20})$ values of "Rosen's postulate" (16).

Determination of HLB parameter

The saturated solutions of therapeutic agents (diclofenac, naproxen, loratadine) obtained after exposure to 25°C in the aqueous solution of solu-

bilizer with $c_{exp} = cmc$ were subjected to condensation at 37°C, dried, and transformed into the solid phase. The 1H NMR spectra of resultant micellar solid dispersions in $CDCl_3$ were obtained as previously described (10, 11). They were used to calculate the hydrophilic-lipophilic balance (HLB) on the basis of the following equation:

$$HLB^{1HNMR} = 15 \times A_h / 0.05(15 \times A_h + 10 \times A_l)$$

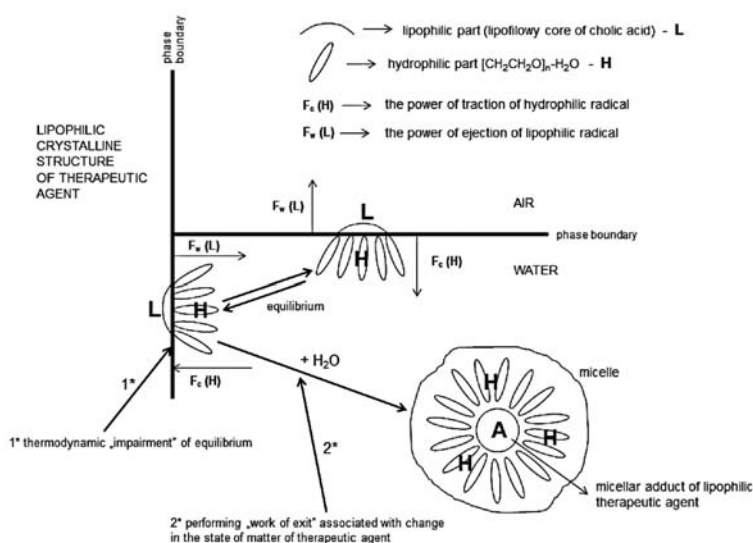


Figure 3. Lipophilic crystalline structure of therapeutic agent

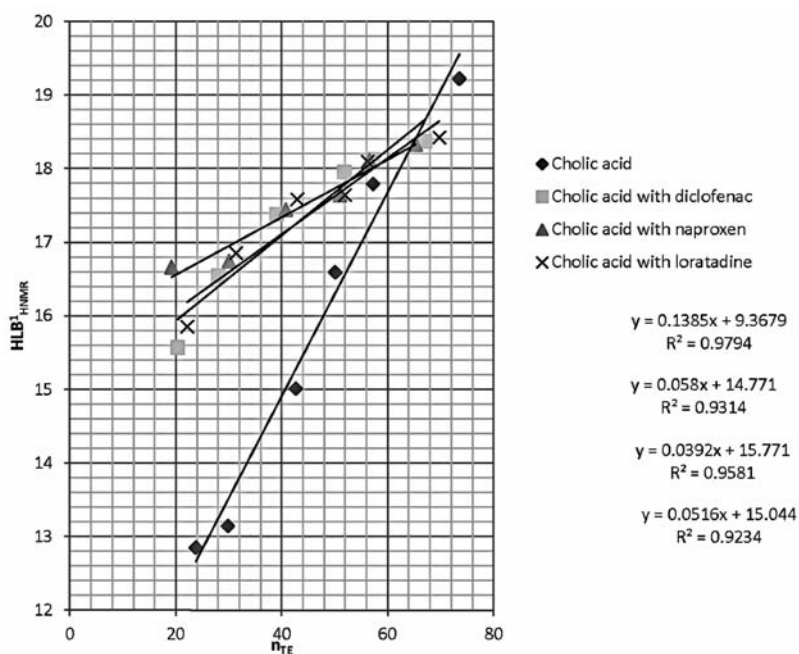


Figure 4. Graphic illustration of relationship: $HLB^{1HNMR} = f(n_{TE})$

Determination of the overall number of lipophilic protons $\Sigma H_1 = 36$ in the structure of the molecule of cholic acid made it possible to calculate the content of oxyethylated segments ($\text{CH}_2\text{CH}_2\text{O}$) in dry micellar adduct after solubilization in equilibrium on the basis of the following equation:

$$n_{\text{TE}} = (36 \times A_h / A_1 - 3) / 4$$

The determined values of n_{TE} and $\text{HLB}^{1\text{HNMNR}}$ are presented in Table 1.

RESULTS AND DISCUSSION

The values of $1/c_{\pi=20}$, $\text{HLB}^{1\text{HNMNR}}$, and n_{TE} presented in Table 1 point to significant variability in the surface activity of the micellar adduct of solubilizer with diclofenac, naproxen, and loratadine, as well as to the variability of ΔG_m^0 , the thermodynamic potential of micelle formation, in relation to the exposure solution of derivative of the oxyethylation of cholic acid with $n_{\text{TE}} = 20\text{--}70$ (Table 1). The n_{TE} and $\text{HLB}^{1\text{HNMNR}}$ of the solubilizer and its micellar adduct also suggest individual variability in the value of "Rosen's postulate", $\log 1/c_{\pi=20}$.

We analyzed the relationship between $\log 1/c_{\pi=20}$ and the determined number of oxyethylated segments, n_{TE} (Table 1, Fig. 1), to estimate the character of thermodynamic interactions occurring at phase boundary. Correlation equations describing the abovementioned relationship at $p = 0.05$ are presented in Table 2. The directional coefficients of the correlation equations (Table 2) allowed us to calculate the value of transport energy to the phase

boundary of the lipophilic part of solubilizer, $[\text{F}\uparrow]$ ($\Delta G_{\text{tr}(l)}$), as well as the value of the transport energy of hydrophilic oxyethylated segment, $[\text{F}\downarrow]$ ($\Delta G_{\text{tr}(h)}$), using the following formulas:

$$\Delta G_{\text{tr}(l)} = a/2.303 \times R \times T$$

$$\Delta G_{\text{tr}(h)} = b/2.303 \times R \times T$$

Presented in Table 2 values of the energy of transport $\Delta G_{\text{tr}(l)}$ and $\Delta G_{\text{tr}(h)}$, in all conditions described by Loughlin allowed us to calculate the ratio:

$$H/L(\Delta G_{\text{tr}}) = n_{\text{TE}} \cdot \Delta G_{\text{tr}(h)} / \Delta G_{\text{tr}(l)}$$

which can constitute our thermodynamic interpretation of hydrophilic-lipophilic balance at phase boundary. The results are presented in Table 3.

The calculated values of $H/L(\Delta G_{\text{tr}})$ ratio (Table 3) suggest that individual progression of this parameter is determined by the method and place of the solubilization of therapeutic agent and significant thermodynamic stability (ΔG_m^0) of micellar adduct. The values of $H/L(\Delta G_{\text{tr}})$ presented in Table 3 inspired us to analyze the relationship $\Delta G_m^0 = f(n_{\text{TE}})$ and to estimate "Rebinder's effect", i.e., the value of energy required to transform the molecule of therapeutic agent from solid body to micelle (molecular state of dispersion). The correlation equations for $\Delta G_m^0 = f(n_{\text{TE}})$ formula, obtained at $p = 0.05$, are presented in Table 4. The directional coefficient "a" of the correlation equation $y = a + b \cdot x$ by $n_{\text{TE}} = 0$ can refer to $\Delta G_{\text{m}(gr)}^0$ of the lipophilic core of cholic acid and its micellar adducts with lipophilic therapeutic agents, i.e., diclofenac ($\Delta G_{\text{m}(gr)}^0\text{D}$), naproxen ($\Delta G_{\text{m}(gr)}^0\text{N}$), and loratadine ($\Delta G_{\text{m}(gr)}^0\text{L}$).

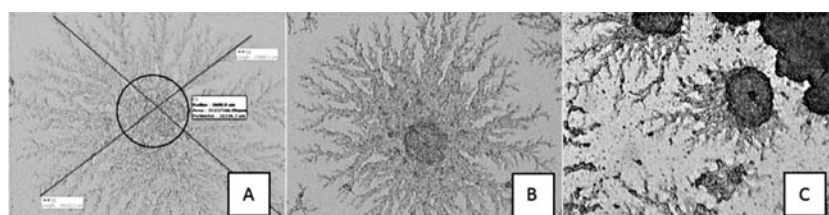


Figure 5. Microscopic structure of the solutions of solubilizers with $n_{\text{TE}} = 70$ and the solutions of adducts after the loss of volatile components (evaporation of solvent): A – solution of surfactant, B – adduct with diclofenac, C – adduct with loratadine

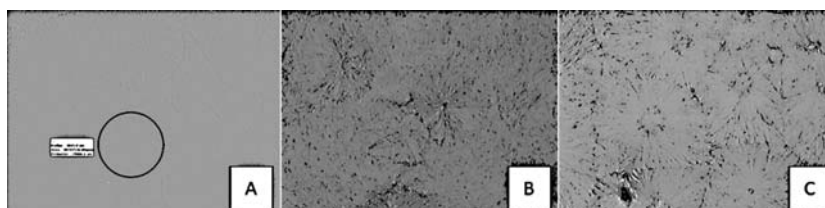


Figure 6. Microscopic structure of the solutions of solubilizers with $n_{\text{TE}} = 50$ and the solutions of adducts after the loss of volatile components (evaporation of solvent): A – solution of surfactant, B – adduct with diclofenac, C – adduct with loratadine

In such a state, due to the proposed experimental model, one can estimate the level of energy (or work) of “Rebinder’s effect” required to transport the molecules of therapeutic agent (following complete moistening) from the surface of solid body to micellar structure, and to form the thermodynamically stable adduct. The values of $\Delta G_{m(gr)}^0$ coefficients presented in Table 4 allowed us to estimate the energy of transport required to change the state of matter on the basis of the following formula:

$$\Delta(\text{“Rebinder’s effect” eR}) = \Delta G_{m(gr)}^0 D,N,L - \Delta G_{m(gr)}^0 \text{ of cholic acid}$$

The values of eR calculated on the basis of the abovementioned formula equaled to:

$$\text{for diclofenac: } \Delta \text{“eR”} = -0.1776 \text{ kJ/mol}$$

$$\text{for naproxen: } \Delta \text{“eR”} = -0.2823 \text{ kJ/mol}$$

$$\text{for loratadine: } \Delta \text{“eR”} = -0.8003 \text{ kJ/mol}$$

The thermodynamic stability of solubilizer’s micelle and the adduct with lipophilic therapeutic agents at phase boundary (air/water) was confirmed by the values of H/L(ΔG_{tr}) ratio (Table 3) and the relationship $HLB^{IHNMNR} = f(n_{TE})$ presented in Figure 4.

On the basis of the abovementioned relationships, we observed that the solubilization of lipophilic therapeutic agent is associated with an increase in the hydrophilicity of the adduct (obtained in solid phase). This was suggested by the values of the directional coefficients of correlation equations presented in Table 4, which varied significantly depending on solubilizers, i.e., the oxyethylation products of cholic acid with $n_{TE} = 20\text{--}70$. We revealed that the products (solid dispersions) obtained upon the micellar solubilization of diclofenac, naproxen, and loratadine in equilibrium, and subsequent dehydration (drying at 37°C) have HLB^{IHNMNR} , which suggests their significant hydrophilicity, irrespective of being the adducts of the lipophilic therapeutic agents.

Analyzing the microscopic structure of compounds obtained upon the loss of the volatile components of the solutions of solubilizer with $c_{exp} \geq c_{mc}$ and the saturated solutions of adducts, we found the important material explanation for the abovementioned statement (Figs. 5, 6) as the therapeutic agents were located in the core, that is to say, in the central part of solubilization space (micelle).

CONCLUSIONS

1. Analyzing an array of homologous structures of cholic acid oxyethylation products with $n_{TE} = 20\text{--}70$, as well as their micellar adducts with lipophilic therapeutic agents: diclofenac, naproxen, and loratadine, we confirmed the relationship

between “Rosen’s postulate” [$\log(1/c_{\pi=20})$] and the content of oxyethylated segments (n_{TE}). The calculated values of transport energy, $G_{tr(h)}$ and $G_{tr(l)}$, constituted the base for determining H/L(G_{tr}) ratio, being the thermodynamic characteristic of hydrophilic-lipophilic balance at the phase boundary.

2. The values of $\Delta G_{m(gr)}^0$ for solubilizer and its micellar adducts with diclofenac, naproxen, and loratadine, which were calculated with approximation formulas, constituted the basis for estimating “Rebinder’s effect” associated with the energy required to transform the molecule of therapeutic agent into the state of molecular dispersion. The value of $\Delta G_{m(gr)}^0$ points to thermodynamic stability and to the possibility of estimating the energy of transport associated with changing the state of matter.
3. The structural character of interaction occurring at phase boundary between the micellar solution of solubilizer and solid body (dispersed therapeutic agent) was confirmed by the values of $\log(1/c_{\pi=20})$, $\Delta G_{m(gr)}^0$, HLB^{IHNMNR} , and H/L(G_{tr}), as well as by the documentation obtained after evaporating water from saturated solution formed as a result of the solubilization of lipophilic therapeutic agent in equilibrium.
4. The results of this study confirm the possibility of comprehensive utilization of “Rosen’s postulate” for the evaluation of solubilization properties of cholic acid oxyethylation products. Moreover, they allow for the thermodynamic estimation of „Rebinder’s effect” associated with the energy of transport required for changing the state of matter of lipophilic therapeutic agent. The results of the research facilitate the application of Rosen’s proposal for the assessment of the usefulness of solubilizers and the stability of the systems obtained.

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TENOXCAM-KOLLIKOAT IR® BINARY SYSTEMS: PHYSICO-CHEMICAL AND BIOLOGICAL EVALUATION

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Abstract: Tenoxicam (TNX) binary systems in Kollicoat IR (KL) matrix were prepared in different drug : polymer ratios using kneading and spray-drying method. The prepared binary systems were characterized for drug dissolution rate, differential scanning calorimetry (DSC), IR spectroscopy and x-ray diffractometry. The results showed that the drug dissolution rate was remarkably enhanced by incorporating it in the KL matrix either by kneading or spray-drying, and the dissolution rate was increased by decreasing the drug weight ratio. The DSC and x-ray studies revealed the presence of TNX in less crystalline or amorphous state in its-KL binary systems. Moreover, the spray-dried TNX-KL system in 1 : 4 ratio, that exhibited the faster dissolution rate, was formulated in oral disintegrating tablets (ODTs). The data indicated that a fast disintegration and higher drug dissolution rate was achieved in case of the ODTs containing the spray-dried form compared to the ODTs containing untreated drug or the commercial tablet (Epicotil®). Also, the drug exhibited significantly ($p < 0.01$) faster onset of the anti-inflammatory analgesic activities in case of the ODTs containing the spray-dried form, that was superior to that observed with both the commercial tablet product and the ODTs containing untreated drug.

Keywords: tenoxicam, Kollicoat IR, spray-drying, kneading, oral disintegrating tablets

Tenoxicam (TNX) is a member of the non-steroidal anti-inflammatory drugs. It inhibits the biosynthesis of prostaglandins by inhibiting the cyclooxygenase pathway. It is also considered as an effective anti-inflammatory agent and has been used in the management of rheumatic and inflammatory diseases, including osteoarthritis (1). However, TNX is very slightly water soluble drug and, as with all poorly soluble drugs, its dissolution may be the rate determining step in the absorption process.

The enhancement of the dissolution rate and solubility of poorly soluble drugs is connected with the application of auxiliary substances or with new technological possibilities (2). Solid dispersions of drugs in water-soluble carriers have attracted considerable interest as a means of improving the dissolution rate, and hence possibly bioavailability, of a range of hydrophobic drugs (3). In such systems, drug undergoes particle size reduction and the consequent increase in the surface area results in the improved dissolution (4), break up the crystal lattice (5) or increasing drug wettability by surrounding hydrophilic carriers (4).

Among the techniques to prepare a solid dispersion, spray-drying has the ability to produce

spherical and size controlled particles and simultaneously to improve the dissolution properties (6).

The dissolution rates of several drugs have been improved by spray-drying with hydrophilic polymers, including indomethacin (7), tolbutamide (8), carbamazepine (9), and ketoprofen (10).

Kollicoat IR is a poly(vinyl alcohol)-poly(ethylene glycol) graft copolymer (PVA-PEG) (11). El-Badry et al. (12) showed that Kollicoat IR-omeprazole microparticles prepared using spray- and freeze-drying techniques revealed the transformation of omeprazole from crystalline to amorphous state with increasing its dissolution rate nine times in comparison to the rate of the physical mixture. Also, Janssens et al. (13) showed that itraconazole was dispersed on a molecular level in the Kollicoat IR microparticles prepared by spray-drying, and showed enhanced dissolution.

Due to the high melting point of Kollicoat IR (210°C) and its poor solubility in the organic solvents, it will be difficult to prepare solid drug-Kollicoat dispersions in its matrix either by melting or solvent evaporation methods.

The present study aims at the preparation of TNX-KL binary systems either by spray-drying or

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kneading and the physicochemical characterization of such systems. In addition, oral disintegrating tablets of an improved TNX-KL binary system will be prepared and characterized in comparison with the untreated drug and the commercial tablet product.

EXPERIMENTAL

Materials

Tenoxicam was kindly supplied by Egyptian International Pharmaceutical Industries Co., EIPICo (Cairo, Egypt). Kollicoat IR (KL) was obtained from BASF (Ludwigshafen, Germany). Microcrystalline cellulose (Avicel PH101) was purchased from Serva Feinbiochemica (Heidelberg, Germany). Spray-dried mannitol (Mannogem™ EZ), used as a filler for the orally disintegrating tablets, was kindly supplied by SPI (Grand Haven, USA). Crospovidone (CPV) was kindly supplied by Riyadh Pharma (Riyadh, KSA). Magnesium stearate was purchased from Riedel-de Haën (Seelze, Germany). Epicotil® tablets (immediate release oblong scored film coated tablets, weighing 200 mg and containing 20 mg TNX), batch number 1108632, was produced by EIPICo (Cairo, Egypt). Carrageenin was purchased from Sigma Chemical Co. (USA). Other materials and solvents are of reagent or analytical grade, and they were used without further purification.

Methodology

Preparation of tenoxicam-Kollicoat IR binary systems

Since Kollicoat is insoluble in most organic solvents, it was not possible to prepare TNX solid dispersions with it using coevaporation method. Therefore, TNX-KL binary systems in different drug : polymer ratios (1 : 1, 1 : 2 and 1 : 4) were prepared either by spray-drying from aqueous solution or kneading methods.

Kneading method

TNX-KL physical mixture was kneaded with appropriate amounts of water (0.1 mL of distilled water for each gram of physical mixture) using a mortar and pestle for 10 min. The mass was dried (room temperature overnight), crushed, sieved and dried again in an oven (Heraeus, Germany) (40°C for 24 h).

Spray-drying

Kollicoat solution was prepared by dissolving the polymer in distilled water in different concentra-

tions (0.5, 1 and 2%) according to the selected TNX : Kollicoat ratios. To the polymeric solution, the weighed amount of TNX was added and dispersed. Thereafter, 1–2 mL of 30% ammonium hydroxide solution was added to raise the solution pH to about 8–9. The clear polymeric solution of TNX was then spray-dried using Büchi 190 mini spray drier (Büchi Labortechnik AG, Germany) with 0.5 mm nozzle. The drug-polymer solution was fed to the nozzle through a peristaltic pump in a spray flow rate of 5 mL/min. The solution was sprayed under the effect of compressed air force (air flow rate of 4 pound per square inch) with an aspiration rate of 100%. The sprayed droplets were dried to remove the solvent in drying chamber by the blown hot air (inlet air temperature of 150°C and outlet air temperature of 80°C). Finally, the resulting dried product was collected from vessel, weighed and stored in tightly closed amber glass containers pending further investigations.

Physical mixture

TNX-KL physical mixtures in different drug weight ratios were prepared by gentle mixing of the weighed amounts the drug and carrier in porcelain mortar.

Characterization of tenoxicam-Kollicoat IR binary systems

In vitro dissolution studies

The *in vitro* dissolution experiments from its KL binary systems and ODTs were performed using USP dissolution apparatus 2, paddle method, (Caleva Ltd., Model 85T), at 100 rpm using a continuous automated monitoring system. This system consists of an IBM computer PK8620 series and PU 8605/60 dissolution test software, Philips VIS/UV/NIR single beam eight cell spectrophotometer Model PU 8620, Epson FX 850 printer, and Watson-Marlow peristaltic pump using in each flask a 900 mL phosphate buffer, pH 6.8. The temperature was maintained at $37 \pm 0.5^\circ\text{C}$. Twenty milligrams TNX or equivalent amount was spread over the dissolution medium. At predetermined time intervals, absorbance was recorded automatically at 362 nm and the percentage of TNX dissolved was determined as a function of time in triplicates.

Dissolution efficiency (DE%) was calculated from the area under the dissolution curve at time t (measured using the trapezoidal rule) and expressed as percentage of the area of the rectangle described by 100% dissolution in the same time (14). Also, the relative dissolution rate (RDR₁₅) data of the different samples were calculated by determining the amount

of TNX dissolved from a particular sample and normalizing for the amount of drug dissolved from pure drug sample over the same time interval (15 min).

In case of oral disintegrating tablets, the same previous conditions were adopted, but the dissolution experiments were carried out on six tablets.

Solubility studies

The aqueous solubility of TNX in its KL binary systems was investigated as follows: an excess amount of TNX or TNX-KL binary system was added to 10 mL of distilled water in a 50 mL glass stoppered bottle. The bottles were firmly closed and placed into the mechanical shaking water bath previously adjusted at $37 \pm 0.1^\circ\text{C}$. After equilibration has been attained (24 h), one mL aliquot sample was withdrawn from each tested solution and diluted to an appropriate volume with distilled water. The absorbance was measured at 362 nm against a suitable blank similarly treated and the drug concentration was calculated

Scanning electron microscopy (SEM)

Morphological characteristics of certain TNX-KL spray-dried and kneaded systems compared to the individual components were observed by scanning electron microscopy (SEM). The samples were sputter-coated with a thin gold palladium layer under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. Coated samples were then scanned and photomicrographs were taken with an SEM (Jeol JSM-1600, Japan).

Differential scanning calorimetry (DSC)

DSC scans were recorded for TNX-KL binary systems compared to that of the individual components in order to determine the extent of crystallinity of the drug in the presence of the studied polymers.

The samples (3–5 mg) were hermetically sealed in aluminum pans and heated at a constant rate of $10^\circ\text{C}/\text{min}$, over a temperature range of 25

to 250°C . Thermograms of the samples were obtained using differential scanning calorimetry (DSC-60, Shimadzu, Japan). Thermal analysis data were recorded using a TA 50I PC system with Shimadzu software programs. Indium standard was used to calibrate the DSC temperature and enthalpy scale. N_2 was used as purging gas at rate of 30 mL/min.

X-ray diffraction analysis

The x-ray diffraction patterns of the powder samples were obtained using RIGAKU diffractometer (Japan), which was equipped with curved graphite crystal monochromator, automatic divergence slit and automatic controller PW/1710. The target used was CuK α radiation operating at 40 kV and 40 mA ($\lambda_{\text{CuK}\alpha} = 1.5418 \text{ \AA}$). The diffraction patterns were achieved using continuous scan mode with $2\theta^\circ$ ranging from 4 to 60° .

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of TNX, KL and their binary systems were recorded using FTIR Perkin Elmer spectrophotometer (Spectrum BX). Samples were mixed with potassium bromide (spectroscopic grade) and compressed into disks using hydraulic press before scanning from 4000 to 600 cm^{-1} . The data were analyzed using Perkin Elmer software (Spectrum V5.3.1).

Preparation of TNX oral disintegrating tablets (ODTs) by direct compression method

TNX ODTs were prepared by direct compression method and the composition of the prepared ODTs containing TNX is listed in Table 1.

The corresponding amounts of TNX, avicel pH 101 and superdisintegrant (CPV) were accurately weighed and mixed using Turbula mixer (Erweka, S2Y, Heusenstamm, Germany) for 5 min. Thereafter, the corresponding amount of mannitol was accurately weighed, added to the mixture and mixed for further 5 min. Finally, the amount of magnesium stearate was mixed with the powder in the Turbula mixer for further 2 min. The powder was compressed into tablets weighing 200 mg using Korsh single punch machine with 9 mm flat punches (Erweka, EKO, Germany).

Evaluation of ODTs containing TNX

Weight variation

Twenty tablets were weighed individually (analytical balance, Shimadzu, EB-3200D, Kyoto, Japan) and the average tablet weight and standard deviation were calculated.

Table 1. Composition of TNX ODTs formulation.

Ingredient	Weight (mg)
TNX (or spray-dried TNX-KL equivalent to 20 mg TNX)	20
Crosspovidone (CPV)	10
Spray-dried mannitol	50
Magnesium stearate	2
Avicel PH101	To 200 mg

Thickness

Pre-weighed 10 tablets were tested for thickness using a micrometer (Mitutoyo M 110-25, Japan), the average thickness and standard deviation were calculated.

Hardness

Tablet hardness of 10 tablets of known weight and thickness was measured using a hardness tester (Pharma test GmbH, Hainburg, Germany). The average hardness and standard deviation were reported.

Friability

Tablet friability was determined according to USP30-NF25. In brief, 20 tablets were weighed (W_1) and placed into the friabilator (Erweka, TA3R, Heusenstamm, Germany), which was rotated at 25 rpm for 4 min. The tablets were then reweighed after removal of fines (W_2), and the loss % was calculated by:

$$100 \times (W_1 - W_2)/W_1$$

In vitro disintegration

In vitro disintegration test was assessed according to the USP30-NF25 requirements. One dosage unit was put in each of the six tubes of the basket (Electrolab, ED-21, Mumbai, India). The apparatus was operated, using phosphate buffer, pH 6.8, as the immersion fluid, which was maintained at $37 \pm 0.5^\circ\text{C}$. Time for complete disintegration of each table, standard deviation and relative standard deviation were calculated.

In vivo studies

Anti-inflammatory activity

The anti-inflammatory activity of TNX ODTs compared to the commercial product (Epicotil®) was evaluated using carrageenin-induced paw edema model (15). All studies were in accordance with the Guidelines of Animal Ethical Committee of King Saud University and had its approval.

The experiment was conducted on 25 albino rats of both sex weighing 90–120 g fasted for 18 h with water available *ad libitum*. They were equally and randomly allocated in 4 groups (6 rats per group). The first group was the rats received ODTs containing untreated TNX; the second group received ODTs containing spray dried TNX-KL system (1 : 4); the third group received the commercial tablet product (Epicotil®) and the fourth group was considered as control.

The rats were anesthetized with urethane (0.5 mL, intraperitoneal) and 100 μL of 1% w/v carrageenin physiologic solution was injected subcutaneously into the treated area. One hour later, a definite weight of the tested tablet containing TNX dose (20 mg/kg) was dispersed in 5 mL of distilled water and immediately given to the rat by an esophageal tube.

Edema volume was measured using a plathysmometer, at suitable time intervals (0.5, 1, 2, 3, 4 and 5 h).

The anti-inflammatory activity (% response) was calculated according to the following equation (16):

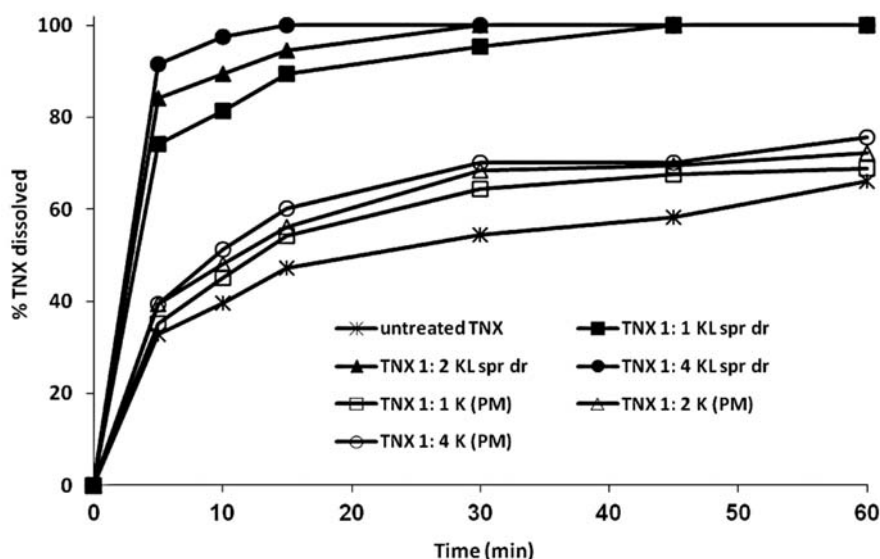


Figure 1. Dissolution profiles of TNX-KL spray-dried (spr dr) systems compared to the corresponding physical mixtures (PM)

Table 2. Dissolution efficiency percentages after 15 min (DE%₁₅), relative dissolution rate after 15 min (RDR₁₅) and aqueous solubility of TNX from its-KL binary systems.

System	DE% ₁₅	RDR ₁₅	Solubility* (mg/mL)
TNX	31.97	-	0.087
TNX-KL spray dried (1 : 1)	66.82	2.06	0.733
TNX-KL spray dried (1 : 2)	73.65	2.18	0.754
TNX-KL spray dried (1 : 4)	79.67	2.30	1.109
TNX-KL Kneaded mixture (1 : 1)	52.51	1.94	0.717
TNX-KL Kneaded mixture (1 : 2)	63.54	1.96	0.88
TNX-KL Kneaded mixture (1 : 4)	66.54	2.20	0.950
TNX-KL Physical mixture (1 : 1)	35.84	1.25	0.102
TNX-KL Physical mixture (1 : 2)	38.60	1.30	0.109
TNX-KL Physical mixture (1 : 4)	40.27	1.41	0.145

*Determined at 37°C

$$\% \text{ Response} = \frac{C - T}{C} \times 100$$

where C = the volume of right paw minus volume of left paw for control rat and T = volume of right paw minus volume of left paw for treated rat.

Hot-plate analgesic test

The analgesic test of the drug ODTs was evaluated using hot-plate method in mice. Mice were divided into 4 groups (6 mice/group). The first group was the mice received ODTs containing untreated tenoxicam; the second group received ODTs containing spray dried TNX-KL system (1 : 4); the third group received the commercial tablet product (Epicotil®) and the fourth group was considered as control. The temperature of the hot-plate metal surface was kept constant at 54 ± 1.0°C. A specific weight of each tablet formulation containing the required drug dose for mice (20 mg/kg) was dispersed in 2 mL of distilled water and administered immediately *via* an esophageal tube. The

time taken by the animals to lick the fore or hind paw or jump out of the place was taken as the reaction time. Latency to the licking paws or jumping from plate was recorded by a stop watch before and after treatment. A latency period of 30 s was defined as complete analgesia cut off time to prevent damage to mice (17).

Statistical analysis

The data from each treatment group were analyzed using an analysis of variance test to determine the p-value for different variables. The Fisher's least significant difference test was used to determine significant differences between two variables.

RESULTS AND DISCUSSION

Characterization of TNX-KL binary systems

In vitro dissolution

Figure 1 shows the dissolution profiles of TNX-KL spray-dried systems in different drug :

polymer ratios (1 : 1, 1 : 2 and 1 : 4) compared to the corresponding physical mixtures as well as the untreated drug. It is clearly evident that TNX showed slow dissolution rate, in which only 47% of the amount was dissolved after 15 min, and has a dissolution efficiency value DE% of 31.97% after 15 min (Table 2). The incorporation of TNX in the matrix of KL during spray-drying resulted in a pronounced enhancement of drug dissolution rate by increasing the polymer weight ratio and a complete drug release was recorded for 1 : 4 ratio after 10 min. The calculated data of DE% after 15 min for the spray-dried systems 1 : 1, 1 : 2 and 1 : 4 were 66.82, 73.65 and 79.67%, respectively. Also, the drug RDR values after 15 min were 2.06, 2.18 and 2.3, respectively. Comparatively, concerning TNX-KL kneaded mixtures, the enhancement of TNX dissolution rate was slightly less than that obtained in case of spray-dried systems (Fig. 2). The recorded DE% after 15 min were 52.51, 63.54 and 66.54% for 1 : 1, 1 : 2 and 1 : 4 kneaded systems, respectively, and the RDR values were 1.94, 1.96 and 2.20, respectively (Table 2). Fouad et al. (18) showed that the increased dissolution rate of celecoxib in spray-dried mixtures of KL IR and other excipients was due to improving the wettability of the drug particles, by significantly reducing the drug particle size during the formation of the SD, or by the inherently higher dissolution rate of the soluble component of the SD introducing the less-soluble component as finely divided particles into the dissolution medium. Other investigators support these finding (19–21).

The dissolution rate of TNX from its KL physical mixture using the same drug : polymer ratios were slightly enhanced compared to the noticeable

increase in the dissolution rate when dispersed in KL matrix by spray-drying or kneading. This might be due to the hydrophilic nature of KL in the physical mixtures.

The solubility of untreated TNX in water was calculated to be 0.087 mg/mL, while its solubility in spray-dried systems was enhanced noticeably by increasing the polymer weight ratio (Table 2). For example, the drug solubility in case of TNX-KL 1 : 4 spray-dried form was 1.09 mg/mL, while it was 0.73 and 0.75 mg/mL in case of 1 : 1 and 1 : 2 ratios. In addition, the solubility improvement of TNX was more pronounced in case of spray-dried binary systems than in case of kneaded ones. Moreover, very slight improvements were recorded for the solubility of TNX in its KL physical mixtures.

Scanning electron microscopy (SEM)

Scanning electron micrographs of TNX, KL, spray dried TNX-KL (1 : 4) mixture and kneaded TNX-KL (1 : 4) mixture are displayed in Figure 3. TNX showed regularly shaped crystals, while KL particles appear highly spherical. The SEM images of spray dried TNX-KL (1 : 4) revealed the presence of very small spheres with regular shapes with no evidence of TNX crystalline shapes. The spherical shapes of the spray-dried TNX-KL with small particle sizes might be one of the factors that are responsible for enhancing drug dissolution and solubility by providing large surface area in addition to surrounding drug particles by the hydrophilic KL particles. However, the kneaded TNX-KL (1 : 4) system appeared as irregular aggregates with large sizes. These data are in accordance with the *in vitro* dissolution data, which proved that spray-dried TNX-KL

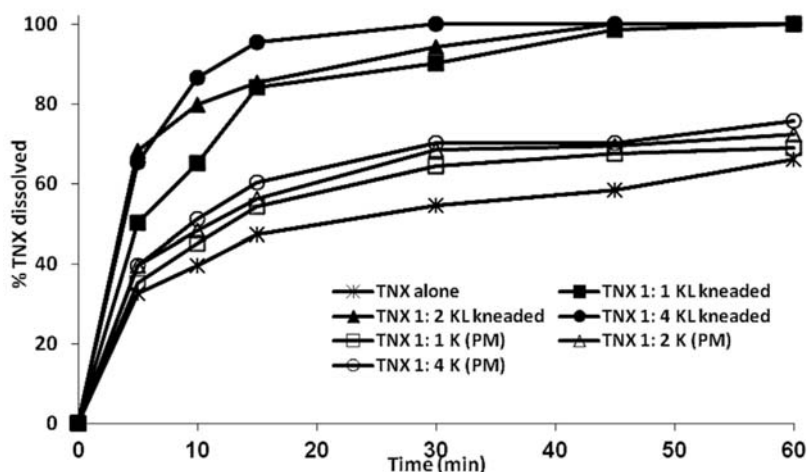


Figure 2. Dissolution profiles of TNX-KL kneaded mixtures compared to the corresponding physical mixtures (PM)

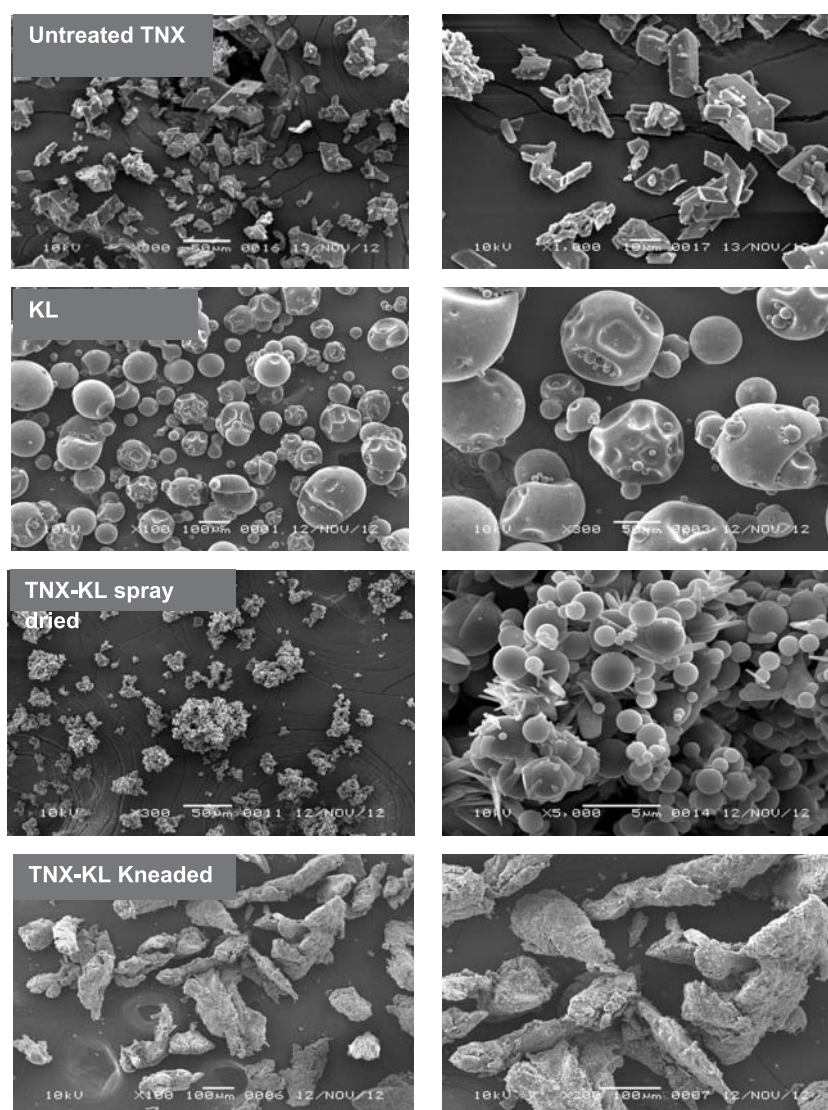


Figure 3. Scanning electron micrographs of TNX-KL spray dried and kneaded systems in drug : polymer ratio 1 : 4 compared to the individual components

improved both drug dissolution rate and aqueous solubility in comparison to the corresponding kneaded form.

The pronounced change of the particles shape in spray-dried mixture may indicate the presence of a new solid phase (22, 23).

Differential scanning calorimetry

Figures 4A and B show the DSC scans of TNX-KL kneaded systems and TNX-KL spray-dried systems in different drug weight ratios compared to the individual components. The DSC curves of TNX show an endothermic peak at 215.9°C with a thaw point at 214°C and heat of fusion, DH, of -47.88 joule/g at a scanning rate of

10°C/min. This endothermic peak ends with an exothermic peak at 220°C, which may be due to the decomposition of the drug when reaching its melting point as reported (24). Kollocoat IR exhibits a broad endotherm at 213.14°C with a thaw point of 207.5°C.

The DSC scans of TNX-KL spray-dried systems (in drug : polymer ratios of 1 : 1, 1 : 2 and 1 : 4), compared to the drug and polymer scans, are displayed in Figure 4A. The drug endothermic peak completely disappeared in all tested TNX-KL spray-dried mixtures, and only the polymer peak was observed at 200°C. This might be due to the solubility in the melted polymer (25). In addition, the drug exothermic peak was shifted to lower temperatures

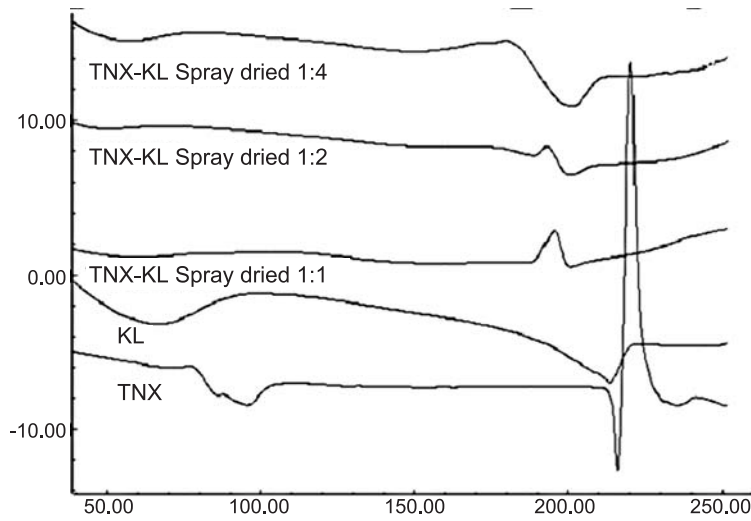


Figure 4A. DSC thermograms of TNX-KL spray-dried systems in different drug : polymer ratios compared to the individual components

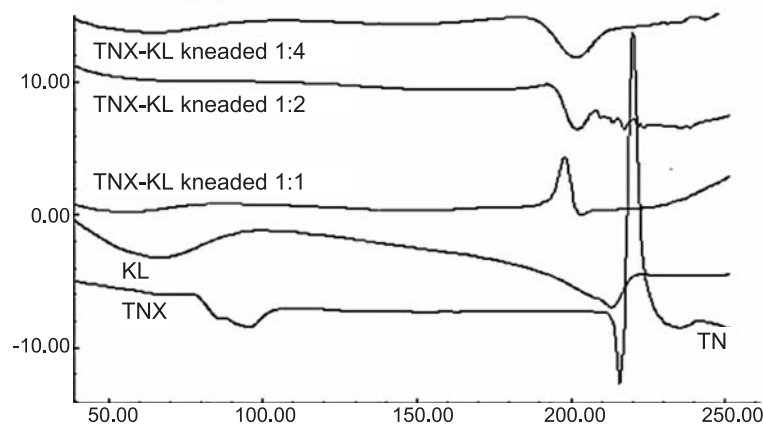


Figure 4B. DSC thermograms of TNX-KL kneaded systems in different drug : polymer ratios compared to the individual components

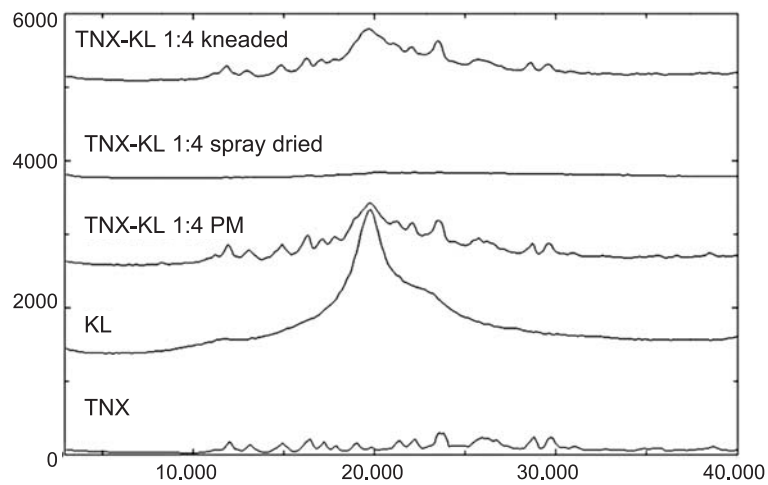


Figure 5. X-ray powder diffraction pattern of TNX-KL spray-dried and kneaded systems in drug : polymer ratio 1 : 4 compared to the corresponding physical mixture (PM) and individual components

Table 3. Physical properties of TNX ODTs (mean \pm SD) compared to the commercial product (Epicotil® tablets).

Tablet formulation	Weight (mg)	Disintegration time (s)	TNX Content (mg)	Hardness (Kp)	Friability (%)	T ₅₀ (s)
ODT containing untreated TNX	205 \pm 5.34	148 \pm 5.91	19.82 \pm 0.89	6.45 \pm 0.25	0.84 \pm 0.19	89.70
ODT containing spray dried TNX-KL (1 : 4)	207 \pm 6.45	23 \pm 1.34	20.12 \pm 0.76	5.98 \pm 0.45	0.86 \pm 0.21	35.73
Epicotil®	200	155 \pm 8.49	20.14 \pm 0.84	–	–	120

(the peak was shifted to 199.4 and 192.9°C in case of spray-dried 1 : 1 and 1 : 2 systems, respectively). However, this exothermic peak was no more seen in the spray dried system at a drug : polymer ratio 1 : 4. Similar results were obtained in case of TNX-KL kneaded mixtures (Fig. 4B). The drug endothermic peak disappeared and the polymer endotherm has been detected in all tested drug : polymer ratios. Also, the exothermic decomposition peak of TNX was shifted to lower temperatures in case of 1 : 1 and 1 : 2 ratios (201°C), and disappeared completely by increasing polymer weight ratio 1 : 4. The characteristic endothermic peak of TNX in its-polymer spray-dried or kneaded mixtures was almost disappeared or reduced in intensity, shifted to lower temperatures and lost its sharpened distinct appearance. The obtained data, in combination with x-ray and scanning electron micrograph findings, confirm the presence of TNX in an amorphous form in these mixtures (26). El Badry et al. (12) found that total drug amorphization of omeprazole was induced in its Kollicoat IR® spray-dried form and these results confirmed that the drug was no longer present in crystalline form and it was changed to amorphous state.

The disappearance of TNX exothermic peak in its-Kollicoat systems (either spray-dried or kneaded) might indicate increased drug stability after kneading or spray-drying (12).

X-ray powder diffraction

To get further evidence on the solid state changes, x-ray diffraction spectra were carried out on TNX, TNX-KL binary systems (1 : 4 weight ratio of drug : polymer) compared to the individual components. The presence of numerous distinct peaks in the x-ray diffraction spectrum of TNX indicates that the drug is present as a crystalline material with characteristic diffraction peaks appearing at diffraction angles of 2θ at 12Å, 14.9Å, 16.5Å, 23.8Å, 28.8Å and 29.7Å (Fig. 5). The diffraction peaks of

KL could be assigned to the two polymers of which Kollicoat IR is composed: polyvinyl alcohol (PVA) and polyethylene glycol (PEG). Indeed, a reflection at 19.7° 2θ due to the presence of crystalline PVA domains and two reflections, 19° (hidden) and 22.9° 2θ , due to the presence of crystalline PEG domains were observed after extrusion (27). The x-ray diffraction spectra of either TNX-KL physical mixture or kneaded mixture did not show any sign of change in the intensity of the drug characteristic diffraction peaks and each of them is seen as a combined effects of TNX and KL diffraction peaks. However, the diffraction spectrum of spray-dried TNX-KL mixture showed a complete disappearance of both the drug and polymer diffraction peaks indicating the loss of their crystallinity. This finding is in accordance with the data obtained by Janssens et al. (13), who showed that the data obtained from x-ray diffraction studies of itraconazole-Kollicoat IR spray-dried solid dispersions suggested that the crystallinity of itraconazole was washed out in the solid dispersions.

FTIR spectroscopy

Figure 6 demonstrates FTIR spectra of the untreated TNX, KL and TNX-KL binary systems in a drug : polymer ratio of 1 : 4.

The spectrum (A) of the drug shows that it is identical with the reported data (24). According to these data, TNX showed a characteristic broad band at 3447 cm^{-1} , which is assigned for the O-H stretching vibration and two bands at 3155 and 3090 cm^{-1} , which are due to the N-H stretching and aromatic C-H vibrations. In addition, a strong band was observed at 1636 cm^{-1} , which was attributed to the amide carbonyl stretching band (C=O). The FTIR spectrum of KL showed a characteristic band at 3421 cm^{-1} , which is assigned for OH stretching.

The FTIR spectra of TNX-KL of both physical mixture and kneaded mixture did not show any change (in terms of the position or intensity) of

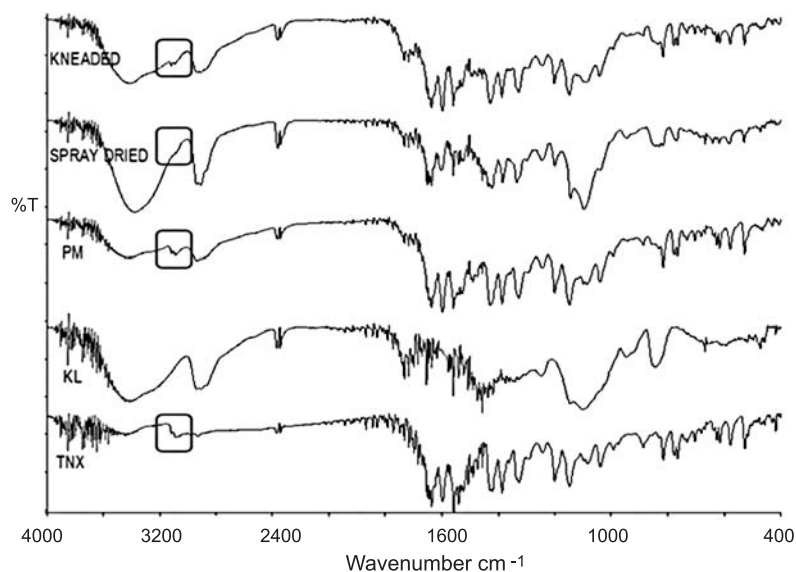


Figure 6. IR spectra of TNX-KL spray-dried and kneaded systems in drug : polymer ratio 1 : 4 compared to the corresponding physical mixture (PM) and the individual components

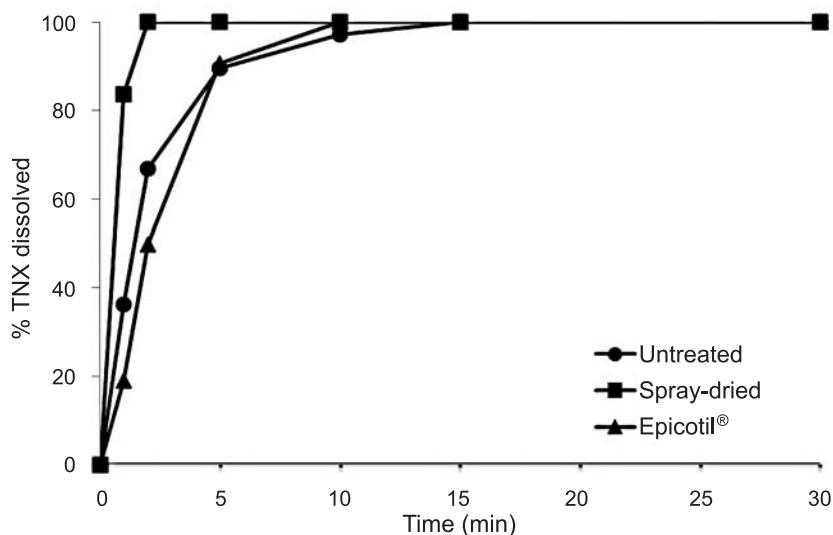


Figure 7. Dissolution profiles of TNX from ODTs formulations compared to the commercial product

either the drug or the polymer characteristic bands. In contrast, the analysis of spray-dried TNX-KL spectrum exhibited complete disappearance of the drug NH stretching band. Additionally, the N-H and O-H stretching bands of TNX and O-H stretching band of KL were all combined as a broad one and shifted to a lower frequency 3368 cm^{-1} . This might

suggest the interaction of TNX and KL in the spray-dried mixture.

TNX oral disintegrating tablets

Tablet evaluation

The oral disintegrating tablets containing 20 mg TNX were successfully prepared using direct

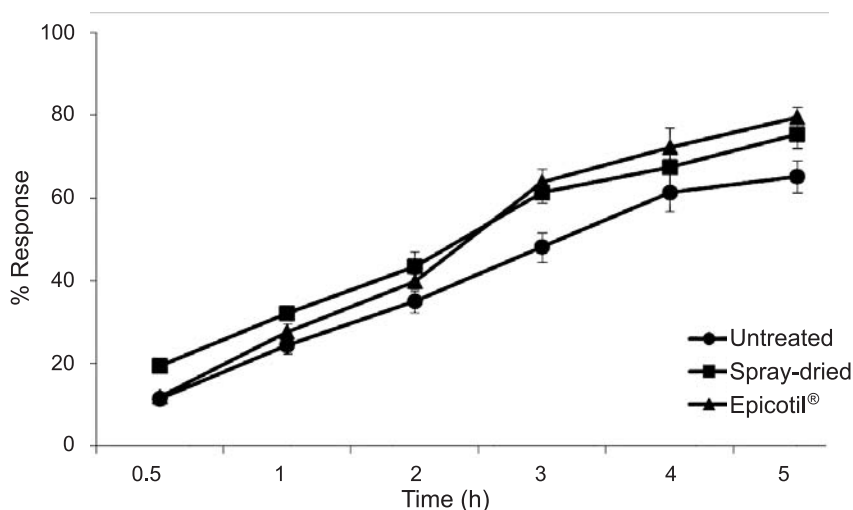


Figure 8. The % anti-inflammatory response of TNX from ODTs formulations compared to the commercial product

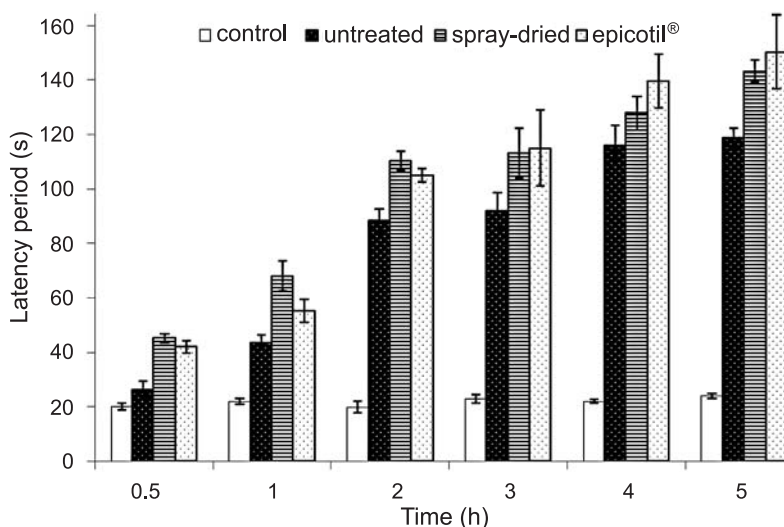


Figure 9. The analgesic activity (represented by latency period in seconds) of TNX from ODTs formulations compared to the commercial product

compression method. The manufactured ODTs were evaluated for their physical properties (weight variation, hardness, friability and drug content), and the obtained data are displayed in Table 3. The weight of the manufactured ODTs containing untreated drug was 205 ± 5.35 mg, while ODTs containing spray-dried drug have a weight of 207 ± 6.45 mg. Moreover, the tablets exhibited acceptable friability that is less than 1% in all ODTs formulations, in addition to acceptable hardness.

In vitro disintegration

The prepared oral disintegrating tablets formulation containing TNX were investigated for their *in vitro* disintegration and compared to the commercial tablet product. Tablets containing spray-dried TNX-KL system (1 : 4) showed fast and complete disintegration within 23 s, while in case of ODTs containing untreated drug, 148 s were required for complete disintegration (Table 3). In addition, the commercial product (Epicotil® tablets) disintegrated completely within 155 s.

***In vitro* dissolution**

The *in vitro* dissolution of TNX from its ODTs containing 20 mg drug was compared to ODTs containing TNX-KL (1 : 4) spray-dried mixture equivalent to 20 mg of drug. Crosspovidone was used as a superdisintegrant in a concentration of 5%. The prepared ODTs were compared to the commercial product (Epicotil® tablets) in terms of dissolution and disintegration (Fig. 7 and Table 3). Untreated TNX showed 36% release within the first minute, and the T_{50} value was attained within 90 s, while ODTs containing spray dried TNX showed 83% release within the first minute and a complete release after 2 min, with a T_{50} value of 37 s. These data are in complying with the ODTs previous data, in which ODTs containing spray-dried drug exhibited faster disintegration. On the other hand, Epicotil® tablets showed slow dissolution rate within the first 2 min due to disintegration of the coating film. Thereafter, rapid dissolution was observed, in which complete drug dissolution was achieved after 10 min, with a calculated T_{50} value of 120 s.

Biological evaluation

Anti-inflammatory activity of TNX ODTs

Oral disintegrating tablets containing untreated TNX and TNX-KL spray-dried system was evaluated for their anti-inflammatory activity using carrageenin-induced paw edema and the data are displayed in Figure 8. Higher % response (i.e., % response of paw swelling) was observed in case of ODTs containing spray-dried TNX-KL system within the first 3 h, which was significantly ($p < 0.01$) higher than that recorded with either ODTs containing untreated drug or the commercial product. This finding is in accordance with the fast dissolution and disintegration of the tablets containing spray-dried TNX-KL system. Then, the calculated % response observed for the commercial product increased, but there was no significant difference between the % responses observed compared to that observed in case of ODTs containing spray-dried drug. During the whole studying period, ODTs containing untreated TNX showed significantly lower % responses compared to the commercial tablets or the ODTs containing spray-dried drug.

Hot-plate analgesic test

Figure 9 shows the analgesic effect of TNX formulation represented by the latency period in seconds. All tested TNX showed longer latency periods, which were significantly different from the control group ($p < 0.01$). During the first 2 h, the analgesic activity of ODTs containing spray-dried

TNX-KL system was significantly ($p < 0.01$) superior to that of recorded with the untreated drug containing ODTs as well as the commercial product. Thereafter, the analgesic activity of the spray-dried TNX-KL containing ODTs and of the commercial tablet are insignificantly different, however, both are significantly different from the control group and untreated drug containing ODTs. It is worthy to refer that the analgesic activity of TNX in its all formulations persisted for 5 h (study period), which might be due to the long half-life of the drug (28).

CONCLUSION

The incorporation of TNX in the matrix of Kollicoat IR could be utilized in improving drug dissolution rate in addition to stabilizing it against exothermic decomposition. Moreover, the spray-dried TNX-KL binary system (1 : 4) showed faster dissolution rate when incorporated in ODTs and also was superior to the commercial product in enhancing both anti-inflammatory and analgesic activities.

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STABILITY OF CILAZAPRIL IN PEDIATRIC ORAL SUSPENSIONS PREPARED FROM COMMERCIALY AVAILABLE TABLET DOSAGE FORMS

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Abstract: Cilazapril is a drug commonly used in management of heart failure in pediatric population. On pharmaceutical market it is available only in inconvenient for pediatric use tablet dosage forms. Until now, no oral liquid formulation containing cilazapril has been evaluated. Therefore, the aim of this study was to prepare easy to made and palatable 1 mg/mL oral liquid formulation with cilazapril (with consideration of original and generic cilazapril tablet and different packages) and subsequent investigation of physicochemical stability of these suspensions. Formulations were compounded using cilazapril obtained from original or generic cilazapril marketed tablet formulations and Ora-Blend® suspending agent. Stability of prepared suspensions stored in closed amber glass or amber plastic PET bottles in the temperature of 298 K was estimated throughout 28 day shelf-life period. Chemical stability was assessed by HPLC cilazapril stability indicating method. Physical stability was evaluated by appearance, taste, smell, pH and rheological assessments. Cilazapril oral suspensions at concentration of 1 mg/mL demonstrated satisfactory stability over 28 day long storage at room temperature. Cilazapril concentrations remained within acceptable limit ($\pm 10\%$) stored in closed amber bottles made of glass or PET material. Moreover, suspensions physical properties remained unaffected. Cilazapril – Ora-Blend® pediatric oral liquid is easy to made, palatable and stable when stored at room temperature for 28 days. Stability of cilazapril oral liquid remains unchanged while using cilazapril tablets produced by different manufacturers and bottles made of amber glass or PET material.

Keywords: angiotensin-converting enzyme inhibitor, cilazapril, pediatric oral suspension, stability

Cilazapril is a specific, selective and long-acting angiotensin converting enzyme inhibitor, which is widely used in therapy of hypertension and heart failure in adults (1–3). It is one of the angiotensin enzyme inhibitors that is also important and widely used in treatment of congestive heart failure in children population (4, 5). Reported pediatric experience with cilazapril is limited, but owing to those clinical studies we possess the knowledge that cilazapril given once a day at dose 0.04 mg of cilazapril/kg of body weight is effective in treating children. Cilazapril doses should be given orally starting from the small doses increasing to reach the final dosage over 1–2 weeks. Effects of such therapy are satisfactory. Cilazapril in children improves cardiac function, reduces left ventricular overload, left ventricular hypertrophy and improves left ventricular function by decreasing mass and wall thickness of left ventricle. Moreover, these beneficial effects persist long-term without tolerance develop-

ment and are characterized by low adverse effect profile (6, 7).

In case of cilazapril therapy essential disadvantage is the lack of proper dosage formulation for children, which are unable to swallow a tablet. Cilazapril is marketed in form of tablets (0.5, 1.0, 2.5 and 5.0 mg cilazapril content) by original and number of generic manufacturers. Good alternative dosing option would be an oral liquid formulation. Preparing stable medications proper to children is challenging. To develop a pharmaceutical formulation many features must be considered such as: excipients, storage conditions, packing, chemical stability of drug substance and final appearance. All mentioned above features that prepared formulation should possess, contribute to better compliance and palatability (8). Presented study is aimed at preparation of an oral liquid with cilazapril and its stability evaluation, because until now neither pediatric oral compounding formulas nor stability data are avail-

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able in the worldwide scientific literature. Needed drug product should be compatible, stable, efficacious, palatable, easy to administer and well tolerated by fastidious pediatric patient.

Cilazapril solubility in water is low (9). Therefore, it is not possible to achieve cilazapril aqueous solution and the only reasonable liquid dosage form for studied drug seems to be an oral suspension (10). Moreover, according to data obtained in the course of previous authors' study (11), it was stated that cilazapril decomposition proceeds by hydrolysis reaction forming biologically active, nevertheless impossible to absorb cilazaprilat molecule. Though, reasonable would be water avoidance or assurance of such environment that prevents hydrolysis occurrence. Ora-Blend® suspending agent was chosen, because it possesses acidic pH (3.5–5.0), ensured by buffering agents, that is considered to be optimal for stability of most hydrolyzable drugs. Moreover, Ora-Blend® vehicle ensures easy manufacturing procedure. Amber bottles (glass and plastic PET) were chosen, while in pharmacopeia it is suggested to protect cilazapril substance from light (9).

Summing up, the aim of this study was to produce easy to prepare, stable cilazapril oral suspension. Stability in prepared oral liquids was tested in oral suspension prepared utilizing original and generic cilazapril 5 mg tablets after storage in amber

PET bottles and amber glass bottles at room temperature (298 K).

EXPERIMENTAL

Preparation of cilazapril 1 mg/mL suspensions

Original (lot: E0128B01U1, expiry date: 09. 2013) and generic (lot: 010612, expiry date: 12. 2013) cilazapril 5 mg tablets were utilized to prepare oral suspensions. In each case 20 tablets were ground in a mortar to a fine powder. Subsequently, obtained powder was levigated with a small amount of Ora-Blend® (lot: 1125072, expiry date: 03. 2013) vehicle to make a paste. Following portions of vehicle were added to obtain liquid, which was subsequently transferred into graduated cylinder. Finally, after rinsing a mortar with vehicle, prepared oral liquid was filled up to 100 mL with Ora-Blend®. Ora-Blend® is ready-made vehicle, which acts as a suspending and flavoring agent, making oral suspension palatable to children. So prepared suspensions were put in amber glass or plastic PET bottles and stored at 298 K over four weeks time.

Stability study

Prepared oral liquids (original drug – formulation A – Ora-Blend® suspension in glass bottle, original drug – Ora-Blend® suspension in plastic PET bottle, generic drug – formulation B – Ora-Blend®

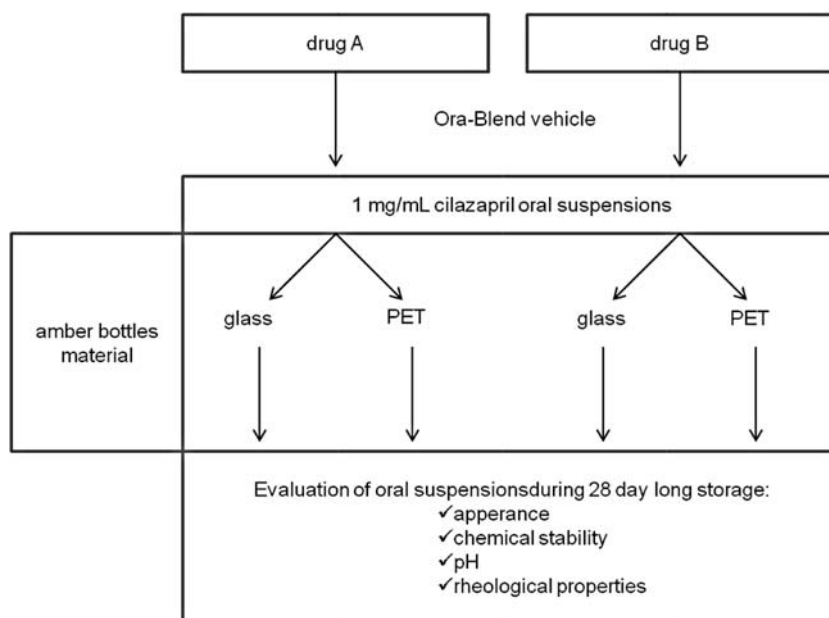


Figure 1. Stability evaluation scheme of cilazapril oral suspensions

suspension in glass bottle, generic drug – Ora-Blend® suspension in plastic PET bottle) were placed in thermostatic chamber ST1+ (Pol-Eko, Poland) with accuracy control 0.1 K set at 298.0 K. Bottles were screwed-capped. Samples were collected on the 0 day, when the suspensions were prepared and on days 7, 14, 21 and 28, during the storage. Before sample collection, each bottle with oral suspension was shaken for 1 min by hand. The samples were assayed by HPLC method with UV detection on the day of sample collection. Specific stability protocol was established for different bottle packs. Bottles used were sterile, in order to avoid microbiological changes in oral liquids and amber, while in pharmacopoeia it is advised to protect cilazapril substance from light (Fig. 1).

Physical stability study

Prepared oral liquids with cilazapril were studied at every sampling for changes in odor by smelling, in favor by spill and spit method and for a change in appearance by vision.

The pH values at every sampling were evaluated by means of calibrated pHmeter MP225 Mettler-Toledo.

Rheological properties of prepared oral suspensions were evaluated on 0 and 28 day of the study. Rheological parameters were determined using rheometer HAAKE RheoStress (Thermo Electro Corporation) equipped with plate-plate measuring system (PP35Ti rheometer RS1 rotor). The system temperature was set at 298 K (thermostat HAAKE DC 30, Thermo Electro Corporation), the same temperature in which oral suspensions were stored for 28 days.

HPLC assay method

The quantitative analysis of cilazapril content in oral suspensions was conducted by means of validated, cilazapril stability indicating HPLC method (12). The Shimadzu HPLC system consisted of: Shimadzu LC-6A liquid chromatograph pump with 7725 Rheodyne valve injector (20 μ L fixed loop), Shimadzu SPD-6AV UV-VIS spectrophotometric detector and Shimadzu C-R6A Chromatopac integrator. Chromatographic separation was achieved on the column: LiChroCART® 250-4 HPLC-Cartridge, LiChrospher® 100 RP-18 (5 μ m) (Merck, Germany). The mobile phase was composed of acetonitrile–methanol–phosphate buffer (pH 2.0) (60 : 10 : 30, v/v/v). The flow rate of mobile phase was set at 1.0 mL/min, column worked at ambient temperature and the injection volume was 20 μ L. The detector wavelength was set at 212 nm. As an inter-

nal standard 0.02 mg/mL oxymetazoline hydrochloride methanolic solution was used.

Samples for HPLC analysis were prepared according to presented below procedure. Volume 2.5 mL of shaken for 1.0 min oral suspension was weighed and transferred quantitatively into 25.0 mL volumetric flask, filled up to the volume with methanol and shaken for 15 min. Subsequently, this suspension was centrifuged at 5800 rpm (MPW-54, MPW) for 10 min. Supernatant was filtered through 0.45 μ L membrane syringe filter. Afterwards, 1.0 mL of obtained solution was mixed with 0.5 mL of 0.02% methanolic solution of oxymetazoline hydrochloride (internal standard). Such solution was subjected to HPLC analysis for cilazapril content.

RESULTS AND DISCUSSION

HPLC method selectivity

The HPLC method with UV detection previously developed and validated for purpose of stability study of cilazapril in solid state (12) turned out to be suitable for evaluation of cilazapril stability in oral suspension made of cilazapril commercial

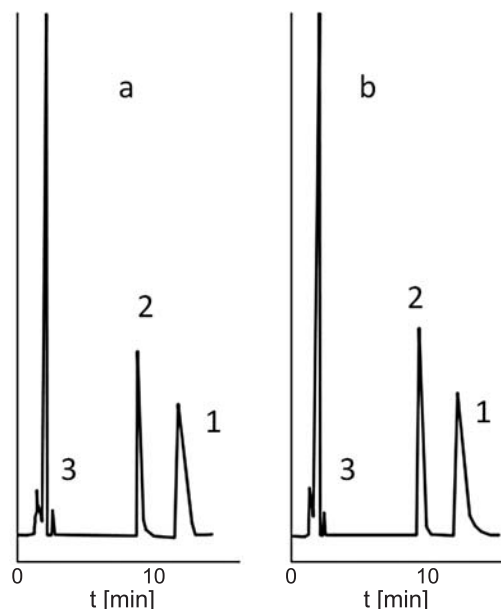


Figure 2. Stability indicating HPLC method, proper for the stability evaluation of cilazapril in Ora-Blend® vehicle. Method is selective towards cilazapril in the presence of excipients of tablet pharmaceutical formulation and contained in Ora-Blend® vehicle. Chromatograms are obtained from analysis of: a) cilazapril oral liquid obtained with original cilazapril tablets (formulation A) and b) cilazapril oral liquid obtained with generic cilazapril tablets (formulation B). Peak (1) match the internal standard and (2) – cilazapril and (3) Ora-Blend® and tablet excipients

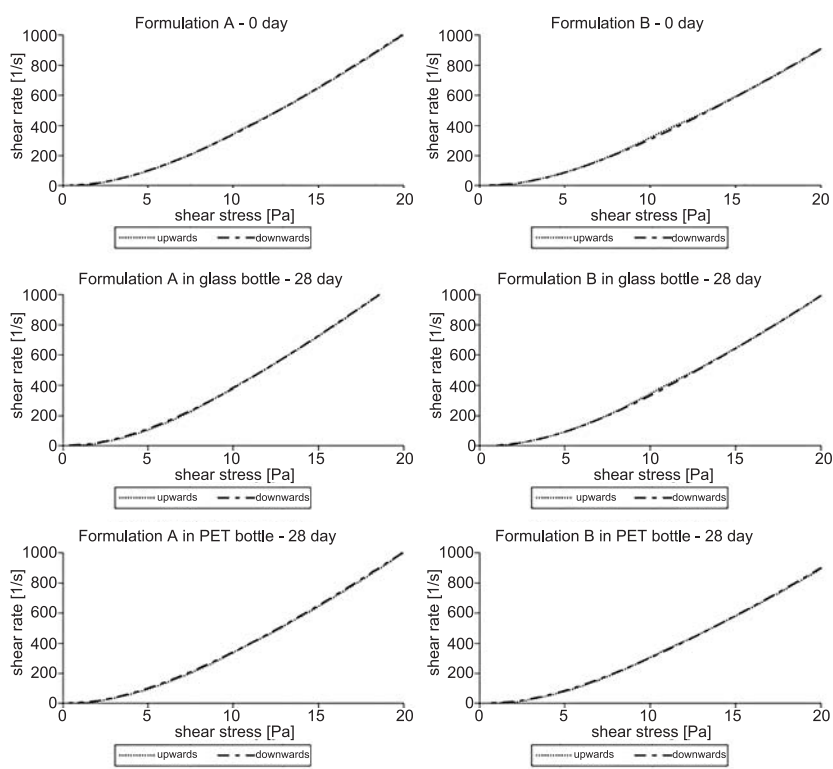


Figure 3. Thixotropic curves of 1 mg/mL cilazapril oral liquids – formulation A and B at 0 day and after 28 day storage at 298 K in amber glass or amber PET bottles

Table 1. Organoleptic properties of 1 mg/mL cilazapril oral suspensions.

Organoleptic properties*	1 mg/mL cilazapril oral suspension	
	formulation A	formulation B
appearance	reddish granular suspension	white granular suspension
taste	berry-citrus and sweet, very slightly bitter	
smell	sweet and fruity	

*No changes in organoleptic properties was observed after 28 day storage at 298 K

tablets (original – formulation A or generic – formulation B). The selectivity of this method was confirmed in the presence of cilazapril tablets and Ora-Blend® excipients. The symmetrical peak of cilazapril, observed at retention time of 8.9 min, was clearly separated from the peaks of excipients and internal standard used (Fig. 2). Throughout the study, degradation of cilazapril occurred, but only in range of $\pm 5\%$ of initial cilazapril concentration (100%), which is acceptable for drug stability in oral suspension. Very low, near the chromatogram base line peak of degradation product appeared during the 28 day long study. It is characterized by reten-

tion time 3.1 min. This retention time is equal with the retention time of cilazaprilat according to authors earlier studies concerning cilazapril stability (11).

Physical stability

Crushed and pulverized tablets of cilazapril (original drug A and generic drug B) were checked for taste by spill and spit method. In both cases taste was unpalatable, bitter and characterized by very unpleasant taste. Ora-Blend® flavors masked unpleasant taste successfully, appearance, and taste of liquid was enhanced.

Table 2. The pH of 1 mg/mL cilazapril oral liquid prepared from original drug tablet (formulation A) and generic drug tablet (formulation B) in Ora-Blend® vehicle stored at 298 K.

Formulation/ Bottle	pH (mean* ± SD)				
	0 day	7 days	14 days	21 days	28 days
A					
glass	4.747 ± 0.005	4.828 ± 0.008	4.727 ± 0.005	4.522 ± 0.004	4.607 ± 0.009
PET	4.747 ± 0.005	4.708 ± 0.086	4.787 ± 0.025	4.537 ± 0.011	4.602 ± 0.017
B					
glass	4.745 ± 0.006	4.838 ± 0.004	4.852 ± 0.024	4.552 ± 0.020	4.647 ± 0.005
PET	4.745 ± 0.006	4.835 ± 0.009	4.540 ± 0.012	4.545 ± 0.013	4.66 ± 0.000

*mean from six independent measurements

Table 3. Rheological properties of 1 mg/mL cilazapril oral liquid prepared from original drug tablet (formulation A) and generic drug tablet (formulation B) in Ora-Blend® vehicle stored at 298 K.

Formulation/ Bottle	K		n	
	0 day	28 days	0 day	28 days
A				
glass	0.39	0.26	0.55	0.62
PET	0.39	0.26	0.55	0.62
B				
glass	0.44	0.26	0.55	0.63
PET	0.44	0.35	0.55	0.59

Table 4. Stability of 1 mg/mL cilazapril oral liquid prepared from original drug tablet (formulation A) and generic drug tablet (formulation B) in Ora-Blend® vehicle stored at 298 K.

Formulation/ Bottle	Percent of initial concentration (mean* ± SD) [%]				
	Initial concentration [mg/mL]	7 days	14 days	21 days	28 days
A					
glass	1.04 ± 0.02	103.24 ± 0.15	101.18 ± 1.54	102.43 ± 1.99	103.13 ± 1.30
PET	1.02 ± 0.01	101.13 ± 0.47	98.43 ± 1.81	100.92 ± 1.10	98.85 ± 0.76
B					
glass	1.01 ± 0.05	98.91 ± 1.98	102.20 ± 3.53	97.14 ± 0.76	99.11 ± 3.74
PET	1.02 ± 0.02	97.81 ± 1.42	99.68 ± 3.52	98.88 ± 3.90	102.43 ± 0.42

*mean from two independent measurements

Prepared oral liquids were acceptable in terms of appearance, smell and taste (Table 1). It is essential, because it contributes to better compliance.

Differences in appearance are caused by different original and generic tablet film coating. Original drug coating is *inter alia* made of red iron dioxide contributing to reddish oral liquid color.

Evaluation of physical properties during stability study of cilazapril oral suspension did not indi-

cate any changes. The flavor, odor or appearance did not seem to change during 28 days of the study.

The pH of suspensions remained stable when stored for given conditions, regardless of tablets used (original and generic) and bottle material. No statistically significant differences at $\alpha = 0.05$ were observed for pH values evaluated during the study (Table 2).

Moreover, pH levels maintained in Ora-Blend® buffering pH range (sodium phosphate monobasic

and citric acid), which is 3.5–5.0. Thus, it can be concluded that buffering system of vehicle ensured stable pH in oral liquid throughout the storage time. Rheological analyses were carried out at the day of oral liquids preparation and after 28 days long storage in amber glass and PET bottles at 298 K temperature. Such parameters as: flow curves, thixotropic curves were evaluated. In all rheological tests oral liquids were subjected to increasing shear rate and, as a consequence, liquid structure was destroyed and liquid started to flow. Nevertheless, when rate shear values decreased, suspension structure was rebuilt, what can be clearly seen on thixotropy curves (Fig. 3). Moreover, from the shape of upwards part of thixotropic curve (the flow curve) it can be concluded that the prepared suspensions are pseudoplastic, shear thinning fluids. Such rheological properties of prepared oral liquids are owing to Ora-Blend® suspending agent. Ora-Blend® is compounded of synergistic blend of suspending agents, which form gel-like matrix counteracting settling down of suspended drug particles.

For mathematical description of studied liquids behavior viscosity Ostwald model was used:

$$\tau = K \cdot \dot{\gamma}^n \quad (1)$$

where τ [N/m] is shear stress; $\dot{\gamma}$ [1/s] is shear rate; n signifies flow behavior index and K fluid consistency coefficient (13). As it results from data presented in Table 3, flow behavior index value < 1 gives evidence that fluid is shear thinning. Changes between K and n parameters observed throughout storage in amber glass or plastic PET bottles through 28 days time in 298 K temperature were minimal and indicated the evidence that 28 day storage did not affect rheological properties of prepared liquids.

Chemical stability

Cilazapril in oral suspensions retained its potency during the storage, while concentration remained within the limits specified in pharmacopoeia ($\pm 10\%$) (Table 4).

CONCLUSIONS

Presented study provides evidence on 28 day stability of cilazapril suspension oral liquid. Stable,

that means that throughout 28 day storage in capped bottles of amber material glass or plastic PET retains within specified limits the same properties as at the time of its manufacture.

Cilazapril oral suspensions in concentration of 1 mg/mL are stable for 28 days in room temperature (298 K), regardless of tablets used (original or generic) and bottle material used.

Acknowledgment

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PHARMACOLOGY

POSSIBLE MECHANISM OF CARDIAC DEPRESSANT ACTIVITY
OF *BERBERIS ORTHOBOTRYS* ROOTS IN ISOLATED RABBIT HEART

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Abstract: *Berberis orthobotrys* Bien. ex Aitch. (*B.o.*) has been reported to have antihypertensive effect in different experimental models. The aim of present study was to evaluate the possible antihypertensive mechanism. Aqueous methanolic extract of *B.o.* roots and its various fractions namely (ethyl acetate, n-butanol or aqueous) in different concentrations (10 ng/mL, 100 ng/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL) were evaluated in isolated perfused rabbit heart to assess their effect on force of contraction, HR and perfusion pressure. The crude extract of *B.o.* and its fractions exhibited a significant decrease in heart rate, contractility and perfusion pressure of isolated rabbit heart, however, butanolic fraction produced more prominent effect and was selected for further study. The effects of butanol fraction were not blocked by atropine (10^{-4} M) in isolated perfused heart. However, butanol fraction significantly blocked the effects of adrenaline (10^{-5} M). It is therefore conceivable that cardiac depressant activity of *B.o.* butanol fraction might be due to the presence of certain β -blocking agents which might be responsible for antihypertensive effect. However, further experiments are required to isolate the active compound(s) and elucidate exact mechanism of action.

Keywords: *Berberis orthobotrys* roots, cardiac depressant, atropine, adrenaline, rabbit

Medicinal plants have always remained a major target for drug development. Most of the traditional medicines used nowadays are from plant origin. In developing countries, medicinal plants provide an alternative therapy, which is cost effective and easily available. It has been estimated that one-third of the world population rely on traditional medicines for their health related needs (1). Traditional medicines though effective yet require evaluation by scientific methods in order to be used to their full extent.

The family Berberidaceae established as “Berberides” is considered one of the most primitive angiosperms (2). Berberidaceae is a small family that contains 15 genera, approximately 650 species worldwide, distributed in temperate regions of northern hemisphere (3). *Berberis* is represented by

3 genera and 20 species in Pakistan. Most of the species are found in mountainous regions of the country. Berberidaceae is characterized by perennial herbs and shrubs, leaves alternate or basal simple or compound, flowers bisexual and actinomorphic (4).

Berberis orthobotrys (Family: Berberidaceae) commonly known as Ishkeen is a plant indigenous to Pakistan, found mainly in Gilgit Baltistan. Its roots and stem bark has been used in the treatment of wounds, infections, piles, jaundice, liver problems, kidney stones, diabetes, sore throat and uterine tumors (5). In our previous study, antihypertensive activity of *B.o.* in rats was evaluated (6). This study was therefore conducted to investigate the possible mechanism of antihypertensive action of *B.o.* in isolated perfused rabbit heart.

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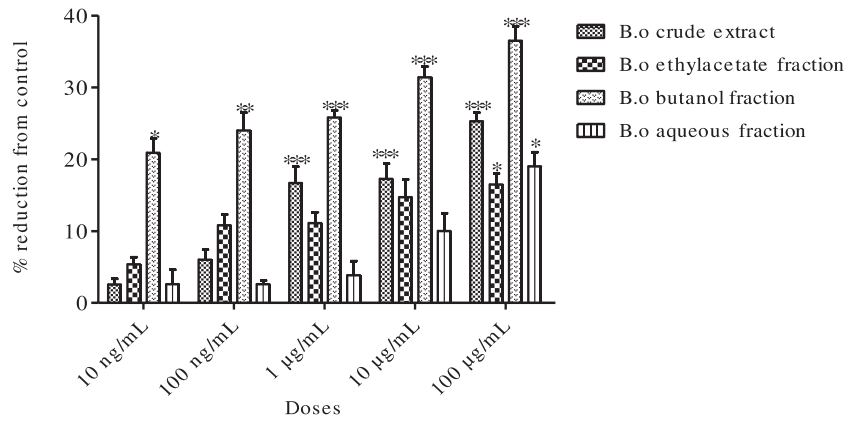


Figure 1. The effect of crude extract of *B.o.* and its fractions on perfusion pressure of isolated perfused rabbit heart (n = 6), where * = (p < 0.05), ** = (p < 0.01), and *** = (p < 0.001) vs. control

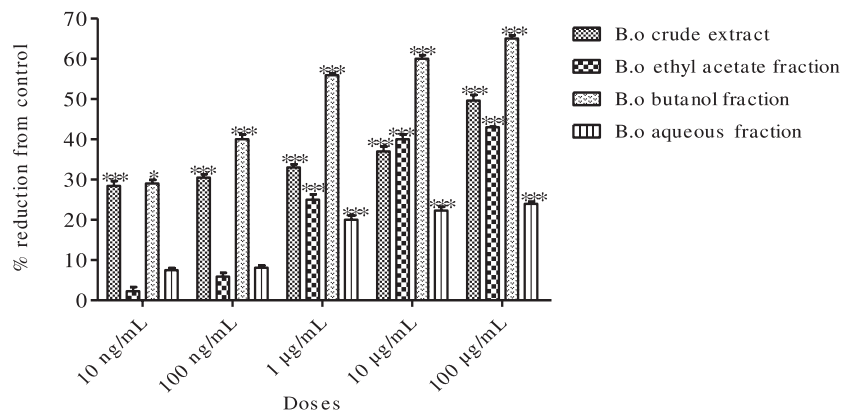
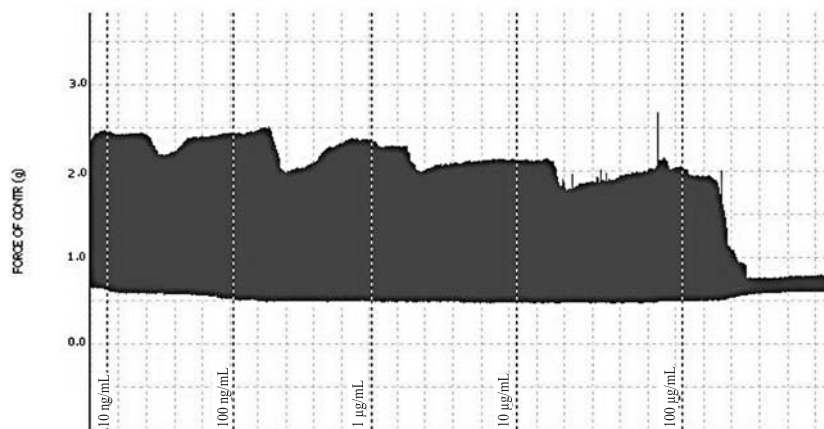


Figure 2. The effect of crude extract of *B.o.* and its fractions on contractility of isolated perfused rabbit heart (n = 6), where * = (p < 0.05) and *** = (p < 0.001) vs. control



Tracing (1) shows the effect of butanol fraction of *B.o.* on contractility of isolated perfused rabbit heart.

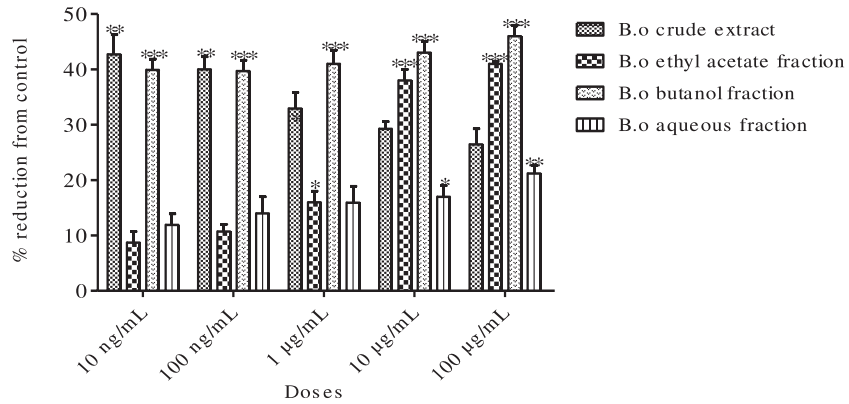
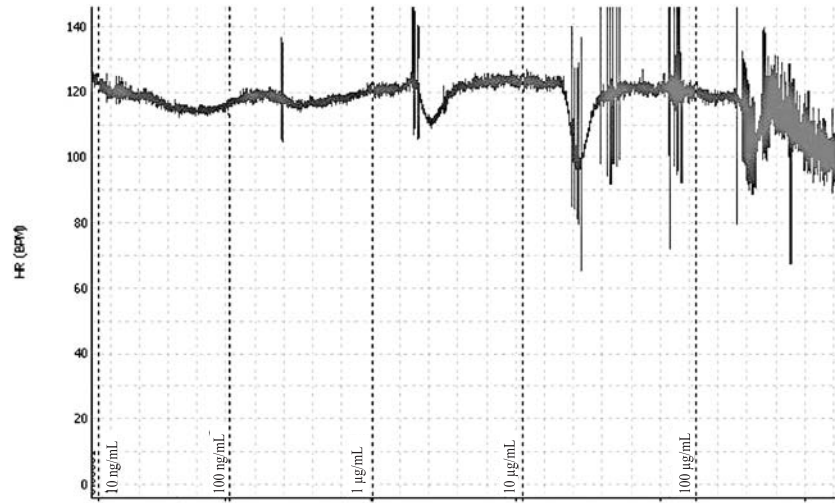


Figure 3. The effect of crude extract of *B.o.* and its fractions on heart rate of isolated perfused rabbit heart (n = 6), where * = ($p < 0.05$), ** = ($p < 0.01$), and *** = ($p < 0.001$) vs. control



Tracing (2) shows the effect of butanol fraction of *B.o.* on heart rate of isolated perfused rabbit heart.

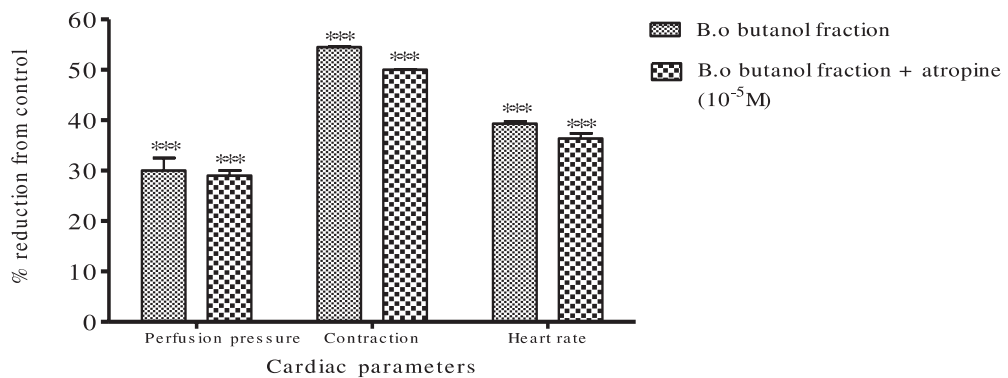
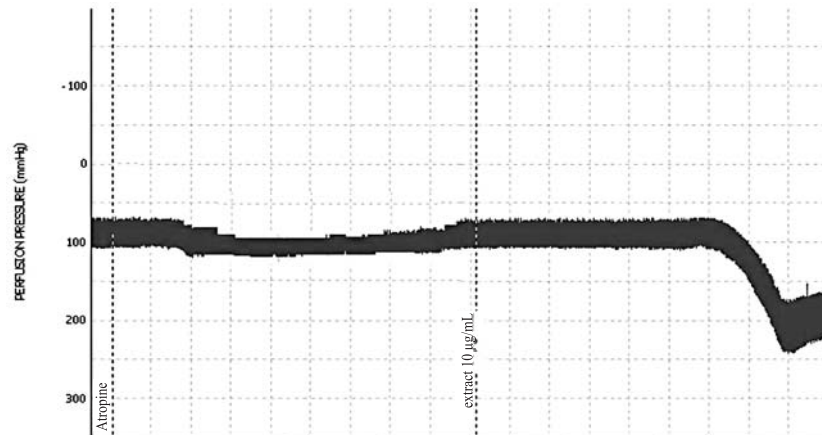
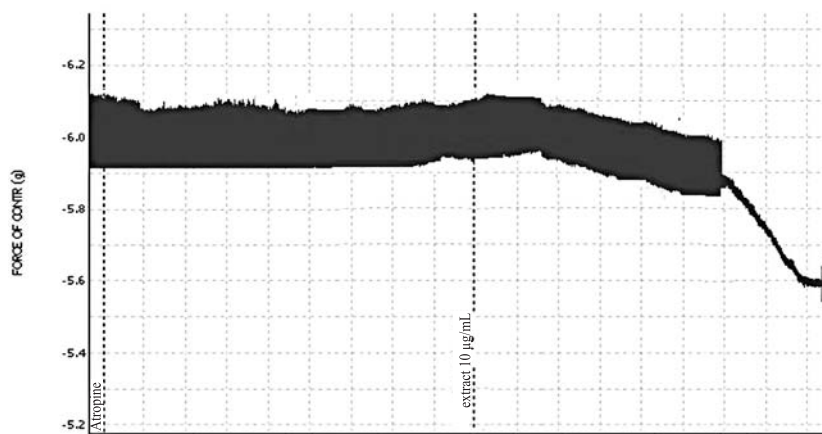


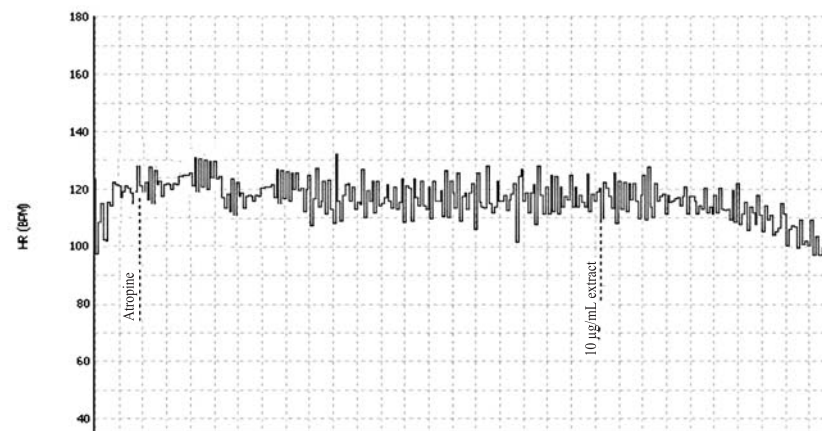
Figure 4. The effect of butanol fraction of *B.o.* (10 µg/mL) on various cardiac parameters of isolated perfused rabbit heart both in the absence and presence of atropine (10^{-5} M) (n = 6), where *** = ($p < 0.001$) vs. control



Tracing (3) shows the effect of butanol fraction of *B.o.* (10 µg/mL) on perfusion pressure of isolated perfused rabbit heart in the presence of atropine (10^{-5} M)



Tracing (4) shows the effect of butanol fraction of *B.o.* (10 µg/mL) on contractility of isolated perfused rabbit heart in the presence of atropine (10^{-5} M)



Tracing (5) shows the effect of butanol fraction of *B.o.* (10 µg/mL) on heart rate of isolated perfused rabbit heart in the presence of atropine (10^{-5} M)

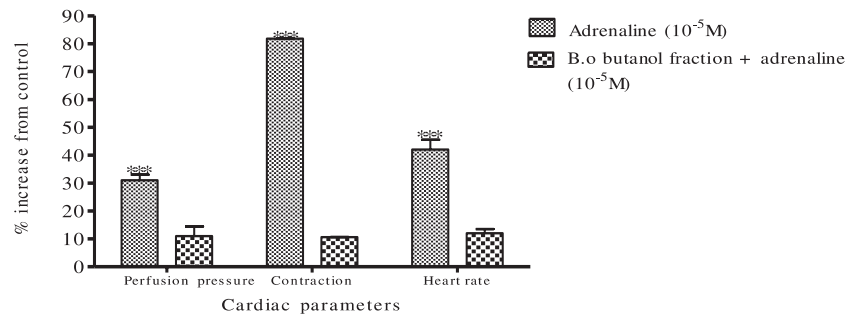
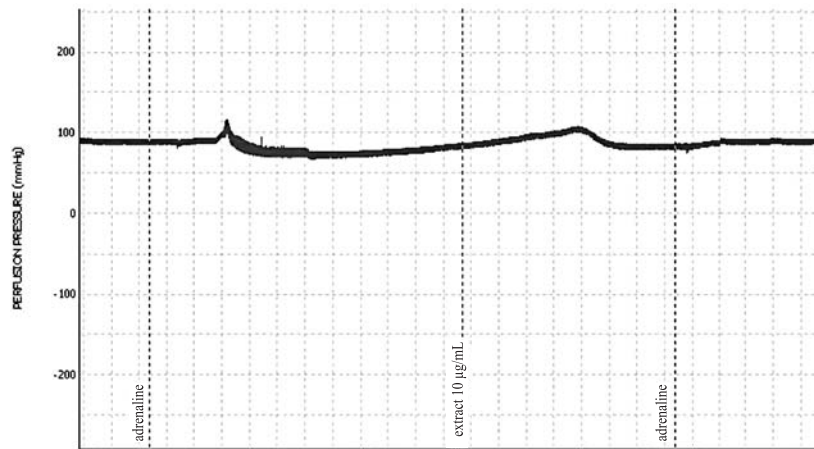
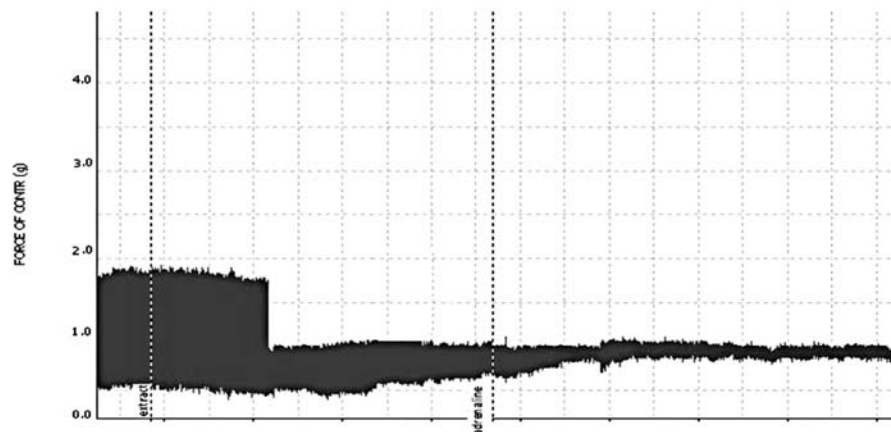


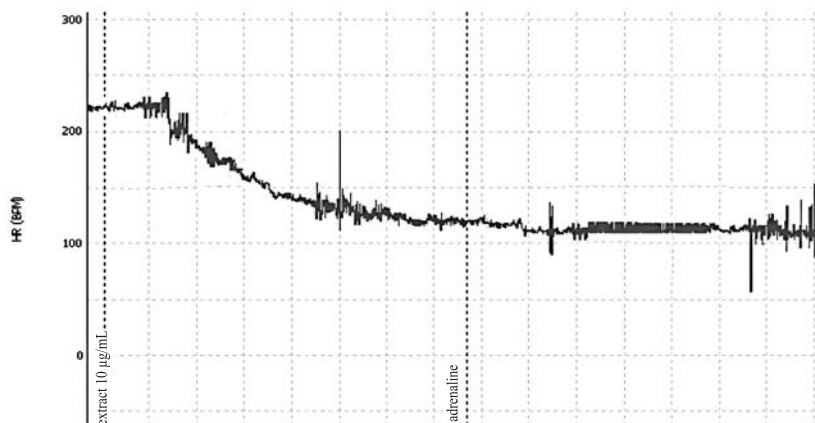
Figure 5. The effect of adrenaline (10^{-5} M) on various cardiac parameters of isolated perfused rabbit heart both in the absence and presence of *B.o.* butanol fraction ($10 \mu\text{g/mL}$) ($n = 6$), where *** = ($p < 0.001$) vs. control



Tracing (6) shows the effect of adrenaline (10^{-5} M) on perfusion pressure of isolated perfused rabbit heart both in the absence and presence of *B.o.* butanol fraction ($10 \mu\text{g/mL}$)



Tracing (7) shows the effect of adrenaline (10^{-5} M) on force of contraction of isolated perfused rabbit in the presence of *B.o.* butanol fraction ($10 \mu\text{g/mL}$)



Tracing (8) shows the effect of adrenaline (10^{-5} M) on heart rate of isolated perfused rabbit in the presence of *B.o.* butanol fraction (10 μ g/mL)

EXPERIMENTAL

Chemicals and drugs

Methanol, atropine and adrenaline were purchased from Sigma Chemicals Co. All the chemicals and drugs used in the experiments were of standard grade.

Animals

Both male and female rabbits of local strain (*Oryctolagus cuniculus*) weighing 1–1.5 kg were used. All the animals were housed in controlled environment (23–25°C) at animal house of Department of Pharmacy, University of Sargodha, Sargodha. All animals were treated according to the standard procedures and the study protocol was approved by the local ethical committee.

Plant material

The roots of *B.o.* were collected from district Gilgit, Pakistan during June, 2011 and were identified and authenticated by Dr. Shair Wali, Assistant Professor of Botany, Karakorum International University, Gilgit, Baltistan, Pakistan. A voucher (no. BO-15-12) has been deposited in the herbarium, Faculty of Pharmacy, University of Sargodha for future reference.

Preparation of extract

Aqueous methanolic (70 : 30) extract of *B. o.* was prepared using cold maceration process. The grounded plant material (2 kg) was soaked in 5 L of water-methanol mixture (70 : 30) for 72 h at room temperature. After three days of occasional shaking,

the whole material was filtered and the filtrate evaporated under reduced pressure using rotary evaporator. The crude extract was then air-dried to obtain a solid mass with a yield of 15% (7).

Fractionation of the extract

Activity directed fractionation of the crude extract of *B.o.* was carried out by using different organic solvents in order to separate or concentrate the activities in anyone of the corresponding fraction. Fractionation was carried out by using different organic solvents (ethyl acetate, n-butanol, aqueous) based on their polarity order, as ethyl acetate < n-butanol < aqueous.

A known quantify of the crude extract was dissolved in distilled water and mixed with equal volume of organic solvents in a separating funnel, shaken vigorously, with periodical removal of air. The mixture was allowed to separate for about 20–30 min into two layers. The respective layer was removed, the same procedure was repeated twice more times. Then, all the fractions were combined and finally concentrated under reduced pressure on rotary evaporator to obtained the corresponding fraction. Similarly, the remaining layer (aqueous) was further treated with other organic solvents for their respective fractions. Finally, the remaining layer was also evaporated and was considered as aqueous fraction (8).

Effect of crude extract and its fractions on Langendorff perfused isolated rabbit heart

This experiment was carried out in accordance with the Langendorff method (9). The rabbit was

injected with 1000 IU of heparin intravenously through the marginal ear vein. Five min later, a blow on the neck of the rabbit made it unconscious. The chest was opened and the heart was dissected out with about 1 cm of aorta attached, and was quickly washed with oxygenated Krebs-Henseleit solution. The isolated heart was gently squeezed several times to remove as much residual blood as possible. The heart was then transferred to the perfusion apparatus (Radnoti isolated heart system, AD Instrument, Australia) and tied to a glass cannula through the aorta. The perfusion fluid was Krebs-Henseleit solution, which was continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide, and was applied at a constant flow mode. The temperature was continuously monitored and kept constant in the range of 36.5–37.5°C.

The heart was allowed to stabilize for 30 min before any drug interventions. The mechanical responses such as force of contraction of spontaneously contracting isolated heart was recorded by attaching one end of a thread to the apex of the heart using a Palmer clip and the other end of the thread to a force transducer (MLT 844; AD Instruments, Australia). Heart rate was calculated indirectly using LabChartpro5 software (AD Instruments, Australia). Perfusion pressure was measured by using pressure transducer. The signals from both pressure and force transducers were filtered and amplified and sent to an analog-to-digital converter (Power Lab data acquisition and analysis system, AD Instruments, Australia) attached to a computer. The signals recorded were saved for later analysis. After stabilization, different doses of the crude extract and fractions (ethyl acetate, n-butanol, aqueous) of *B.o.* (10 ng, 100 ng, 1 µg, 10 µg, 100 µg/mL) were applied to assess various cardiac parameters i.e., heart rate (beats/min), force of contraction (g) and perfusion pressure (mm Hg) with each heart serving as its own control. In order to explore the possible mechanism of action, the effect of butanol fraction of the extract was assessed both in the absence and presence of atropine 10^{-5} M (10). In addition, the effects of adrenaline (10^{-5} M) were also assessed both in the absence and presence of butanol fraction of *B.o.* (11).

Statistical analysis

The results were expressed as the means \pm SEM. Statistical analysis was done by two way ANOVA followed by Bonferroni test using Graph Pad Prism 5.0. Value of $p < 0.05$ was considered as significant.

RESULTS

Effect of crude extract of *B.o.* and its fractions on perfusion pressure, contractility and heart rate of isolated perfused rabbit heart

The aqueous methanolic extract of *B.o.* at all doses produced a significant ($p < 0.01$ – 0.001) decrease in force of contraction of isolated heart while a significant ($p < 0.05$ – 0.01) decrease in heart rate was observed only at doses from 10 ng/mL to 1 µg/mL. The crude extract (1–100 µg/mL) also exhibited a significant ($p < 0.001$) decrease in perfusion pressure. Ethyl acetate and aqueous fractions of *B.o.* demonstrated a significant negative inotropic and chronotropic effect at higher doses only. Similarly, both extracts produced a significant decrease in perfusion pressure merely at 100 µg/mL. Moreover, butanol fraction of *B.o.* produced a significant ($p < 0.05$ – 0.001) decrease in perfusion pressure, heart rate and contractility of isolated heart at all the doses. A prominent effect in all the three cardiac parameters was observed at 10 µg/mL. Butanol fraction was chosen as the most potent of all of the extracts, hence 10 µg/mL of butanol fraction was selected to elucidate its possible mechanism of action. The highest dose of the butanol fraction (100 µg/mL) caused a maximum decrease in contractility and heart rate but it also produced hardening of the heart and stoppage of beating of the heart. Therefore, this dose was not picked to explore its detailed mechanism (Figs. 1, 2 and 3).

Effect of butanol fraction of *B.o.* on perfusion pressure, contractility and heart rate of isolated perfused heart in the presence of atropine (10^{-5} M)

In the presence of atropine (10^{-5} M), the butanol fraction of *B.o.* produced a significant ($p < 0.001$) decrease in perfusion pressure, force of contraction and heart rate of isolated heart. Atropine did not block the negative inotropic and chronotropic effects or reduced perfusion pressure of butanol fraction. The decrease in all the cardiac parameters was quite similar in both presence and absence of atropine (10^{-5} M) (Fig. 4).

Effect adrenaline (10^{-5} M) on perfusion pressure, contractility and heart rate of isolated perfused heart in the presence of butanol fraction of *B.o.*

To find out the possible β -blocking activity, the effects of adrenaline on various cardiac parameters were studied both in the presence and absence of butanol fraction of *B.o.* (10 µg/mL). The findings indicated that adrenaline (10^{-5} M) produced a significant ($p < 0.001$) increase in force of contraction,

heart rate and perfusion pressure of isolated heart in the absence of butanol extract of *B.o.* (10 µg/mL). However, in the presence of butanol extract of *B.o.* (10 µg/mL), the effects of adrenaline (10⁻⁵ M) were significantly blocked as there was non-significant increase in all the cardiac parameters of isolated heart (Fig. 5).

DISCUSSION

Herbs were our first source of medicines. No one knows when humans first used plants for medicine, but pollens of at least six medicinal plants were found in a Neanderthal burial site estimated to be at least 60,000 year old and some derivatives, e.g., aspirin, reserpine, and digitalis, have become mainstays of human pharmacotherapy. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets. Several natural product drugs of plant origin have either recently been introduced in the market, including galantamine, nitisinone, and tiotropium, or are currently involved in late-phase clinical trials (12, 13). The medicinal values of traditional medicinal plants cannot be disregarded and studies have been carried out in order to explore various active principles of the extracts with intensive follow up studies to establish their exact mechanism of action. One of the most important area in which compounds from plant sources have contributed successfully, is the cardiovascular research.

The present study revealed that aqueous methanolic extract of *B.o.* exhibited a significant decrease in perfusion pressure, force of contraction and heart rate at most of the doses. Similarly, all the fractions of *B.o.* produced a significant negative inotropic and chronotropic effect in isolated heart. However, ethyl acetate and aqueous fractions of *B.o.* showed a significant decrease in perfusion pressure only at 100 µg/mL. Butanol fraction of *B.o.* was found to be much more potent than other extracts as it exhibited a significant decrease in all the cardiac parameters; this clearly indicates that the active principle(s) responsible for the said effects were more concentrated in this fraction. Hence, butanol fraction of *B.o.* (10 µg/mL) was selected for a detailed mechanism study.

Previously, it has been reported that negative inotropic and chronotropic effect in isolated heart usually results from a cholinergic/histaminic stimulating activity or by β-receptor or calcium channel blocking effect (14, 15). A decrease in perfusion pressure indicates a coronary vasodilation effect, which is generally caused by three different mediators that include β-receptor antagonist, cholinergic

receptor agonist and endothelium derived relaxing factor (9, 15).

In the presence of atropine (10⁻⁵ M), butanol fraction of *B.o.* (10 µg/mL) produced a significant decrease in perfusion pressure, heart rate and force of contraction of isolated rabbit heart indicating that the extract did not mediated its actions through cholinergic receptors. In order to investigate the β-blocking effect of butanol fraction of *B.o.* (10 µg/mL), the effects of adrenaline (10⁻⁵ M) were studied in isolated rabbit heart. The findings indicated that the butanol extract significantly blocked the positive inotropic and chronotropic effects of adrenaline (10⁻⁵ M). Similarly, the increased perfusion pressure produced by adrenaline (10⁻⁵ M) was also significantly antagonized. It has been well established that adrenaline, a sympathomimetic drug, acts directly on β₁ receptors and produces an increased heart rate and contractility. Propranolol blocks these receptors and produces a negative inotropic effect and chronotropic effect (16, 17). Hence, the blocking of adrenaline's pharmacological effects by the butanol fraction of *B.o.* clearly indicated its β-receptor antagonizing activity on isolated heart. However, the involvement of other receptors cannot be ruled out. Moreover, it has also been reported that β-blockers produce a cardiac inhibitory effect that results in decreased cardiac output and vasodilation, ultimately leading to a fall in blood pressure. Other blood pressure lowering mechanisms of β-adrenergic receptor blocking agents include a decrease in catecholamine release and an antirenin activity (18). It is presumable therefore that this cardiac depressant activity of *B.o.* in isolated perfused rabbit heart might also be responsible for its antihypertensive effect in rats.

CONCLUSION

It is concluded from this study that cardiac depressant and coronary vasodilating activity of *B.o.* might be due to its antagonizing effects on β-receptors. Further studies are required to identify and isolate the biologically active compounds in order to determine the exact mechanism of action.

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PHARMACOLOGICAL EVALUATION OF ANTIHYPERTENSIVE EFFECT OF AERIAL PARTS OF *THYMUS LINEARIS* BENTH.

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Abstract: Traditionally *Thymus linearis* Benth. have been used for treatment of various diseases including hypertension. The present study was conducted to evaluate the hypotensive and antihypertensive effect of aqueous methanolic extract of aerial parts of *Thymus linearis* Benth. in normotensive and hypertensive rats. Acute and subchronic studies were also conducted. The aqueous methanolic extract produced a significant decrease in SBP, DBP, MBP and heart rate of both normotensive and hypertensive rats. LD₅₀ of the extract was found to be 3000 mg/kg. The extract also exhibited a reduction in serum ALT, AST, ALP, cholesterol, triglycerides and LDL levels, while a significant increase in HDL level was observed. It is conceivable therefore, that *Thymus linearis* Benth. contains certain active compound(s) that are possibly responsible for the observed anti-hypertensive activity. Moreover, these findings further authenticate the traditional use of this plant in folklore medicine.

Keywords: *Thymus linearis* Benth., antihypertensive, isolated heart, LD₅₀

Cardiovascular diseases (CVDs) have become a major risk and their prevalence has gradually increased throughout the world. CVDs include coronary heart disease, cerebrovascular disease, hypertension, arrhythmias, rheumatic heart disease and heart failure. Hypertension refers to an increased blood pressure and usually 140/90 mm Hg is taken as threshold. It has been estimated that high blood pressure is responsible for 6% of deaths worldwide and remains the prominent risk factor for cardiovascular diseases affecting millions of people in Pakistan (1). According to National Health Survey of Pakistan, about one hundred thousand deaths occur per year in the country due to CVDs. Many synthetic drugs have been commonly used for the treatment of hypertension in developed countries but herbal medicines still remain the popular choice in the developing countries. The abundant use of these

herbal medicines has led to an extensive research in this area to determine their potential efficacy and several modern cardiovascular drugs are now available as natural/herbal products. Ethnobotanical surveys in our country have indicated their vast use in the treatments of CVDs. For example, hydro-alcoholic extract of the leaves of *Syzygium guineense*, has produced a decrease in blood pressure of hypertensive rats. Similarly, *Passiflora nepalensis* Wall reported to be effective in hypertension. Moreover, the Chinese have used *Ginkgo biloba*, *Stephania tetandra* and *Uncaria rhynchophylla* for the treatment of hypertension (2, 3).

Thymus linearis Benth. (Family: Lamiaceae) locally known as “Tumuro” is a perennial plant found mostly in all alpine and subalpine pastures of Pakistan, India, Afghanistan and Nepal (4). Traditionally it has been widely used to treat a num-

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ber of ailments including high blood pressure, toothache, headache, cold, fever as well as skin, eye, and liver diseases (5, 6). In the present study, we have endeavored to evaluate antihypertensive effects of aqueous methanolic extract of *Thymus linearis* Benth. in both normotensive and hypertensive rats.

MATERIALS AND METHODS

Chemicals

Glucose and methanol were purchased from Sigma Chemicals Co. All the chemicals used in the experiments were of the best analytical grade.

Animals

Both male and female Sprague Dawley rats (200–300 g) and, albino mice (20–25 g) were used. The animals were housed in controlled environment (23–25°C) at animal house of University of Sargodha and were handled according to the standard procedures. The study protocol was approved by the local ethical committee Faculty of Pharmacy, University of Sargodha.

Collection and identification of plant material

The aerial parts of plant were collected from a village shikyote district Gilgit, Gilgit Baltistan, Pakistan during the month of July, 2011 and were identified and authenticated by Dr Shair Wali Assistant Professor, Department of Botany, Karakoram International University, Gilgit Baltistan. A voucher specimen with no. T.L.B=57-11 has been deposited in Faculty of Pharmacy, University of Sargodha for future reference.

Preparation of plant extract

Aqueous methanolic (70 : 30, v/v) extract of aerial parts of *Thymus linearis* Benth. was prepared using cold maceration process. The grounded plant material (2 kg) was soaked in 7 liters of an aqueous methanolic mixture (70 : 30) for 72 h at room temperature. After three days of occasional shaking, the whole material was filtered and the filtrate was evaporated under reduced pressure using rotary evaporator and finally dried in lyophilizer.

Effect of extract of *Thymus linearis* Benth. on blood pressure and heart rate of normotensive rats

Sprague Dawley rats of either sex were used for these experiments and were randomly divided into three groups of five rats each. Group I received

100 mg/kg of the extract of *Thymus linearis* Benth. Group II and Group III were given 250 mg/kg and 500 mg/kg of the aqueous methanolic extract of *Thymus linearis* Benth., respectively. Blood pressure and heart rate of each of these groups were determined at 0 hour, then after 2, 4 and 6 hours after administration of extract by using non-invasive blood pressure (NIBP) measuring apparatus (NIBP Controller – AD Instruments) from the tail of rats. Each animal was placed in the restrainer and an appropriate cuff with sensor was mounted on their tails and then warmed to about 33–35°C. The tail cuff was inflated to a pressure well above the expected systolic blood pressure SBP (200 mmHg) and slowly released during which the pulse was recorded by using Power Lab data acquisition system and computer running Lab chart 5.0 software as described by (3). SBP, MBP and heart rate were measured directly using pulse tracing while the diastolic blood pressure was calculated from SBP and MBP using the equation: $DBP = (3MBP - SBP)/2$.

Effect of aqueous methanolic extract of aerial parts of *Thymus linearis* Benth. on blood pressure and heart rate of cholesterol fed hypertensive rats

Sprague Dawley rats of either sex were randomly assigned into two groups of five rats each. Group I served as control and was treated with a specially prepared egg feed diet in order to produce cholesterol-induced hypertension. The diet was prepared by the addition of yolk of 12 eggs to 500 g standard rat diet. The feed so prepared was dried in the sunlight for 3 days. Animals were then fed on this diet for 21 consecutive days. Group II served as treated group and received egg feed diet and aqueous methanolic extract of *Thymus linearis* Benth. (500 mg/kg) for 21 consecutive days. BP and heart rate of these groups were measured on days: 0, 7, 14 and 21 (7).

Effect of aqueous methanolic extract of *Thymus linearis* Benth. on blood pressure and heart rate in glucose fed hypertensive rats

Sprague Dawley rats of either sex were divided into two groups of five rats each. Group I served as control and received 10% glucose solution for 21 consecutive days. Animals in group II were given 10% glucose solution and aqueous methanolic extract of aerial parts of *Thymus linearis* Benth. (500 mg/kg) for 21 consecutive days. BP and heart rate of each of these groups were measured at 0, 1, 2, and 3 weeks by placing them in a pre-warmed restrainer. SBP, MBP and heart rate were measured directly using pulse tracing while the diastolic blood pres-

Table 1. Effect of various doses of crude extract of *Thymus linearis* on SBP, MBP, DBP and heart rate of normotensive rats.

Time (h)	Doses											
	100 mg/kg				250 mg/kg				500 mg/kg			
	SBP	MBP	DBP	HR	SBP	MBP	DBP	HR	SBP	MBP	DBP	HR
0	127.5 ± 2.44	104.2 ± 0.78	92.5 ± 0.90	383 ± 10.0	125.9 ± 0.49	104.8 ± 0.71	94.3 ± 0.68	378 ± 9.80	125.7 ± 1.00	105.2 ± 0.36	95.9 ± 0.84	388 ± 4.56
2	125.3 ± 2.32	100.9 ± 0.79	90.7 ± 0.68	370 ± 6.62*	122.6 ± 1.62	100.9 ± 0.59	93.5 ± 0.78	356 ± 7.13*	114.5 ± 3.28*	99.2 ± 2.24	92.0 ± 3.75	360 ± 4.70**
4	120.5 ± 4.69	95.0 ± 2.55*	88.7 ± 1.08	365 ± 4.68**	116.1 ± 2.48*	95.3 ± 1.28*	90.9 ± 1.11	350 ± 8.65**	104.2 ± 2.76**	90.9 ± 1.48*	85.7 ± 1.51*	340 ± 5.19***
6	118 ± 1.82**	90.0 ± 3.68**	85.5 ± 0.89	360 ± 8.16**	113.6 ± 2.83**	80.64 ± 2.47**	85.6 ± 0.83*	340 ± 7.25**	100.4 ± 1.6***	86.6 ± 1.60**	77.3 ± 2.75**	320 ± 5.22***

Results are expressed as the mean ± SEM (n = 5). One way ANOVA followed by Dunnett's test have been applied; * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs. control 0 h.

Table 2. Effect of *Thymus linearis* on egg feed-induced hypertensive rats.

Days	SBP (mm Hg)		DBP (mm Hg)		MBP (mm Hg)		Heart rate (beats/min)	
	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg
	0	121.6 ± 3.49	123.8 ± 1.78	89.60 ± 2.17	100.8 ± 2.34	101.0 ± 1.58	108.6 ± 2.07	361.0 ± 5.98
3rd	144.2 ± 3.67	109.2 ± 4.45**	101.4 ± 1.93	90.4 ± 2.45**	114.4 ± 2.48	98.21 ± 2.22*	404.1 ± 3.32	350.1 ± 6.12***
6th	149.4 ± 3.67	100.8 ± 5.97**	107.4 ± 3.83	85.2 ± 1.84**	120.6 ± 3.60	90.40 ± 3.00**	431.0 ± 4.96	320.2 ± 3.78***
9th	168.0 ± 4.57	95.20 ± 3.88***	110.0 ± 4.28	80.6 ± 2.39***	124.4 ± 2.14	83.60 ± 2.23**	455.0 ± 5.47	300.2 ± 4.93***

Results are expressed as the mean ± SEM (n = 5). One way ANOVA followed by Dunnett's test have been applied; * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs. control day 0.

Table 3. Effect of *Thymus linearis* on glucose induced hypertensive rats.

Weeks	SBP (mm Hg)		DBP (mm Hg)		MBP (mm Hg)		Heart rate (beats/min)	
	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg	Control	Treated 500 mg/kg
	0	124.6 ± 2.01	122.6 ± 1.73	87.21 ± 2.79	97.80 ± 2.42	98.01 ± 1.85	108.2 ± 2.36	383.6 ± 4.85
1st	139.6 ± 3.05	102.6 ± 3.50**	93.25 ± 2.62	89.4 ± 2.05*	109.2 ± 2.68	95.60 ± 2.27*	390.4 ± 4.31	371.6 ± 8.18**
2nd	148.4 ± 4.95	99.41 ± 3.94**	102.4 ± 1.71	84.60 ± 2.30*	116.0 ± 2.40	87.61 ± 3.03**	406.2 ± 3.99	317.0 ± 9.10***
3rd	166.4 ± 4.94	82.2 ± 2.60***	112.4 ± 2.07	75.60 ± 1.82**	127.1 ± 2.76	77.00 ± 3.13***	410.4 ± 3.89	296.6 ± 5.49***

Results are expressed as the mean ± SEM (n = 5). One way ANOVA followed by Dunnett's test have been applied; * = (p < 0.05), ** = (p < 0.01), *** = (p < 0.001) vs. control (week 0).

sure was calculated from SBP and MBP using the equation $DBP = (3MBP - SBP)/2$ (3).

Acute toxicity study

Albino mice of either sex were randomly divided into five groups (n = 2). Group 1 served as control and received normal saline (10 mL/kg) while other groups (Groups 2–9) were administered different doses of the extract in an ascending order i.e., 100, 500, 1000, 1500, 2000, 2500, 3000, 3500 mg/kg, respectively. The mortality rate was observed for 24 h. All the doses were administered by intraperitoneal route. The highest dose, which did not kill any animal, and the lowest dose, which killed only one animal, was noted. LD₅₀ was calculated from the geometric mean of these two doses (8).

Subchronic toxicity

Sprague Dawley rats of either sex were randomly divided into two groups (n = 6). The first group received normal saline (10 mL/kg body weight *p.o.*) and the animals in group 2 received 500 mg/kg body weight of the extract daily for 28 days. Food and water intake of animals were observed during this period. At 29th day, blood was collected from overnight fasted rats of each group by cardiac puncture for the determination of serum biochemical parameters. Then, the rats were sacrificed by cervical dislocation for the study of various organs (heart, liver and kidney) weights (9).

Biochemical parameters

For the estimation of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phos-

phatase (ALP), total cholesterol, triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL) blood samples were collected in clot activator gel tubes. The serum was separated by centrifuging the blood samples at 2000 r.p.m. for 10 min. Serum biochemical parameters were then measured by using commercially available reagent kits (9).

Statistical analysis

The results were expressed as the means \pm SEM. One way ANOVA followed by Dunnett's test have been applied with $p < 0.05$ considered as significant.

RESULTS

Effect of *Thymus linearis* Benth. extract on blood pressure and heart rate of normotensive rats

The extract produced a significant decrease in the SBP, MBP, DBP and heart rate with 100, 250 and 500 mg/kg dose especially after 4 and 6 h of drug administration, however, more pronounced effect was produced by 500 mg/kg, hence, it was selected for further study (Table 1).

Effect of extract *Thymus linearis* Benth on blood pressure and heart rate of cholesterol fed hypertensive rats

The extract of *Thymus linearis* Benth, at a dose of 500 mg/kg produced a highly significant ($p < 0.001$) decrease in SBP, MBP, DBP and heart rate of cholesterol fed hypertensive rats. A highly pronounced effect in all the observed parameters

Table 4. Effect of *Thymus linearis* on body weights, organ weights and various biochemical parameters of rats.

Parameters	Control	Extract 500 mg/kg
Liver (g)	5.86 \pm 0.10	5.85 \pm 0.12 ^{NS}
Kidney (g)	1.39 \pm 0.14	1.38 \pm 0.11 ^{NS}
Heart (g)	1.47 \pm 0.11	1.46 \pm 0.12 ^{NS}
Body weight (g)	280 \pm 5.11	281 \pm 4.50 ^{NS}
ALT (IU/L)	0.1 \pm 2.29	36.9 \pm 1.69*
AST (IU/L)	90.1 \pm 1.06	80.2 \pm 1.09*
ALP (IU/L)	70.0 \pm 2.01	58.2 \pm 1.94*
Triglycerides (mg/dL)	89.2 \pm 1.92	75.0 \pm 1.74*
Cholesterol (mg/dL)	60.5 \pm 1.56	45.0 \pm 1.02*
LDL (mg/dL)	20.09 \pm 2.07	14.20 \pm 1.09**
HDL (mg/dl)	33.4 \pm 1.18	46.5 \pm 2.02*

Values are expressed as the means \pm SEM (n = 6); * = $p < 0.05$, ** = $p < 0.01$ and NS = Non-significant as compared to control.

was seen at second and third weeks of treatment (Table 2).

Effect of extract *Thymus linearis* Benth. on blood pressure and heart rate of glucose fed hypertensive rats

The extract of *Thymus linearis* Benth. at a dose of 500 mg/kg produced a highly significant ($p < 0.001$) decrease in SBP, MBP, DBP and heart rate of glucose fed hypertensive rats with more significant effects at week 2 and 3 (Table 3)

Acute and subchronic toxicity studies

The median lethal dose (LD_{50}) of the extract in mice was found to be 3000 mg/kg. In subchronic toxicity studies, the extract did not cause any significant alteration in body weights or organs weights of rats. The results also revealed that the extract (500 mg/kg) produced a significant reduction in serum ALT, AST and ALP levels. The extract exhibited a significant decrease in total cholesterol, triglycerides and LDL levels while increase in HDL levels was observed (Table 4).

DISCUSSION

Plants have been used for the treatment of various diseases since ancient times. In the last few years there has been an exponential growth in the field of herbal medicine and these drugs are gaining popularity both in developing and developed countries because of their natural origin and less side effects (10).

In the present investigation in normotensive and hypertensive rats, the extract produced a significant decrease in SBP, MBP and DBP with maximum effect at 500 mg/kg. Similarly, the extract produced a significant decrease in the heart rate of both normotensive and hypertensive rats. It was observed that the degree of antihypertensive response produced by the extract of *Thymus linearis* Benth. was much higher in hypertensive rats than in normotensive rats. This observation was in support of the general finding that hypertensive animals appear to have an exaggerated response to depressor stimuli (11). A decrease in sympathetic activity is involved in decrease in blood pressure (12). The extract also produced a significant decrease in heart rate of normotensive rats possibly due to a reduction in sympathetic activity, which might have also been involved in a decreased blood pressure.

Previous studies demonstrated that atherosclerosis may contribute to an increase in blood pressure (13). In cholesterol fed hypertensive rats, the extract produced a significant decrease in blood pressure and heart rate. Moreover, the extract also cause a decrease

in LDL, TG, cholesterol and an increase in HDL level, which could be responsible for its antihypertensive effect in cholesterol-induced hypertensive rats.

It has been reported that a high glucose intake cause an increase in blood pressure through the generation of reactive oxygen species (ROS). These ROS production have been found to be associated with an increased NADPH oxidase activity (14). Moreover, it has also been reported that an increased glucose level is involved in the inhibition of nitric oxide synthase activity, ultimately resulting in decreased nitric oxide levels and an increased blood pressure (15). Thus, the antihypertensive effect of the extract in glucose treated hypertensive rats might be due to an inhibition of NADPH oxidase activity or an increased level of nitric oxide.

During subchronic toxicity studies, the extract was found to be safe and no signs of toxicity were observed. Food and water intake, body weights and organ weights remained unaltered during the 28 days of treatment with the extract. However, biochemical parameters related to hepatic functions, such as ALT, AST and ALP, were significantly decreased when compared to control. The reduction of these enzymes indicated that the extract did not cause any toxic effects on both liver and heart tissues (16). The extract also revealed a significant decrease in serum cholesterol, triglyceride and LDL levels in rats as compared to control. The increase in HDL levels and a decrease in LDL levels by the extract indicated a possible reduction in cardiovascular risk factor that could lead to the death of animals. These effects are quite similar to those of anti-hyperlipidemic drugs like statins (17). Therefore, the antihypertensive effect of aqueous methanolic extract of *Thymus linearis* could also be associated with its lipid lowering effects.

CONCLUSIONS

It is concluded from this study that the antihypertensive effect of *Thymus linearis* Benth. aerial part might be due to certain biologically active compounds present in the plant extract. However, further studies are needed to isolate the active antihypertensive principle(s) and assess its possible mechanism of action.

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SAFETY AND EFFICACY OF BONE WAX IN PATIENTS ON ORAL ANTICOAGULANT THERAPY

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Abstract: Cardiovascular conditions, apart from neoplastic diseases, remain the major cause of death in developed countries; therefore, the number of patients receiving oral anticoagulants is constantly increasing. Anticoagulant therapy considerably reduced mortality in patients with history of myocardial infarction among others. Although many interventions may be performed without withdrawal of the anticoagulant and tooth extraction was qualified as a procedure of low hemorrhage risk, a majority of dentists refer the patient to a cardiologist several days before the elective tooth extraction to withdraw anticoagulants. The aim of the study was to evaluate the efficacy and safety of bone wax used to stop bleeding after dental procedures in a group of patients on chronic anticoagulant therapy and find an answer to a question, whether it is justified to temporarily withdraw anticoagulants for this type of procedures. The study involved 176 patients on chronic anticoagulant therapy undergoing tooth extraction (154 subjects) or surgical extraction of a retained tooth (48 subjects). After the procedure, in each case the alveolus was filled with bone wax to stop bleeding. In all patients involved in the study bleeding from the alveolus was successfully stopped during the procedure. None of the subjects reported increased bleeding from the operational site after coming back home. Bone wax is a good, efficient, and safe material to block bleeding from the alveolus following tooth extractions, also in patients on chronic anticoagulant therapy. The study demonstrated that withdrawal or adjustment of anticoagulant therapy is not necessary before an elective tooth extraction.

Keywords: post-extraction bleeding, bone wax, anticoagulants, cardiovascular disease

Many systemic diseases require anticoagulant therapy. The conditions, which should be mentioned here, include ischemic stroke, arterial embolism, pulmonary embolism, atrial fibrillation or preventive treatment of myocardial infarction, as well as many others (1–3). Cardiovascular conditions, apart from neoplastic diseases, remain the major cause of death in developed countries (4, 5). In 2010, the ischemic heart disease led to 12.9 million deaths in the world (4), which constitutes a quarter of the total number of deaths (4, 6).

Introduction of acetylsalicylic acid combined with oral anticoagulants or antiplatelet drugs considerably reduced mortality and the number of subsequent ischemic events in patients with history of myocardial infarction (7–9). In spite of optimal pharmacological treatment as well as compliance with the rules of secondary prevention, several percent of patients annually develop new coronary

events or die (10, 11). Oral anticoagulants require often INR (International Normalized Ratio) testing and consequently, corrections of the drug dose as the INR increase above the therapeutic range raises the risk of hemorrhage complications (1, 2). Sustained INR level of 2–3.5 in patients treated with oral anticoagulants results in low risk of bleeding (1, 12).

The number of patients on chronic antiplatelet and anticoagulant therapy is increasing, which creates a challenge for doctors performing procedures affecting tissue continuity. Due to the risk of hemorrhage, the management algorithm compels doctors to withdraw the drugs or use a bridge therapy for several days before and after the surgical procedure.

In a patient treated with anticoagulants, who is being prepared for an invasive procedure, the risk of major bleeding with procedure-related death must be assessed and compared to the thromboembolic risk caused by temporary withdrawal of drugs (13).

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Many interventions may be performed without withdrawal of anticoagulants to prevent “paying off” a minor invasive procedure with permanent disability (e.g., due to a stroke) or death (13, 14). Tooth extraction was qualified as a procedure of low hemorrhage risk (3, 13, 14) to be performed without the anticoagulant therapy being necessarily withdrawn (1, 15–19). However, most dentists recommend a consultation with a cardiologist as routine management before withdrawing anticoagulants for several days due to elective tooth extraction.

The study was designed to evaluate efficacy and safety of bone wax used to stop bleeding after tooth extraction or surgical extraction in a group of patients on chronic anticoagulant therapy as well as to answer the question whether it is justified to withdraw anticoagulants for this type of procedures, which is established a standard management recommended by dentists.

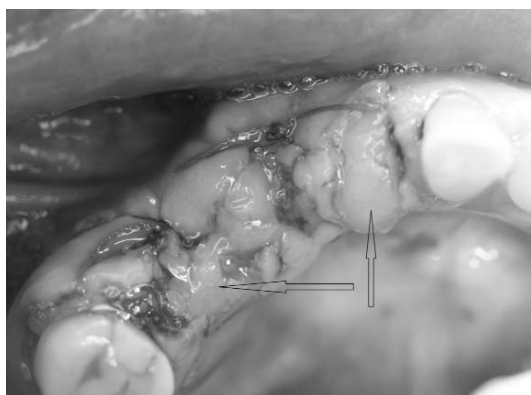


Figure 1. Surgical wax sitted into the alveolus

MATERIALS AND METHODS

The study involved 176 patients reporting for tooth extraction. All of the subjects took oral anticoagulants (Table 1). Patients with a history of myocardial infarction occurring later than 6 months before the procedure, uncontrolled background disease requiring anticoagulant therapy as well as patients receiving intramuscular anticoagulants were excluded from the study.

The study consisted in 154 extractions of erupted teeth and 48 surgical extractions of retained teeth. The procedure was performed under local anesthesia on an outpatient basis. After each tooth extraction or surgical extraction the alveolus was filled with non-absorbable bone wax to seal the interrupted vessels of the bone. The wax consisted of 80% beeswax and 20% isopropyl palmitate. The wound of the mucous membrane was sutured, which prevented the wax from falling out too quickly and closed the space left after the tooth was removed, mechanically blocking bleeding from soft tissues (Fig. 1). The procedures were performed before noon to enable an hour observation of the patient following the procedure.

After 5–7 days of the procedure, patients reported for follow-up and wax removal, if it had not been washed out.

RESULTS

In all the patients involved in the study bleeding from the alveolus was successfully stopped during the procedure. At the follow-up visit none of the subjects reported increased bleeding from the oper-

Table 1. Drugs received by patients qualified for treatment.

	Number of patients	Background disease	Mode of action	Trade name
ANTIPLATELET DRUGS			COX- 1 inhibition	
Acetylsalicylic acid	135	Coronary disease, myocardial infarction, arterial thrombosis in carotid arteries and lower limb arteries, stroke.		Aspirin, Acard, Polocard
Ticlopidine	10	Use: see above when aspirin is contraindicated.		Aclotin
VITAMIN K ANTAGONISTS			vit. K inhibition	
Acenocoumarol	31	Atrial fibrillation, history of venous thrombosis, pulmonary embolism, artificial heart valves.		Acenocoumarol, Sintrom

ational site after coming back home. No case of purulent exudate from the wound was found. In nearly 1/3 of subjects (65 alveoli) the applied bone wax stayed within the site for 7 days and required mechanical removal.

Among the study group, 32 subjects did not report for the follow-up visit at the scheduled date and in 12 cases prolonged presence of bone wax caused local inflammation within the healing wound. No case of allergic reaction was found.

DISCUSSION

Apart from wax, which is the oldest and the cheapest hemostatic material, bleeding control after tooth extraction may be obtained by mouth rinsing with solution of tranexamic acid q.i.d. for 2 days or 25% solution of α -aminocaproic acid (1, 2, 15) or local wound dressing with materials like spongostan, fibrin sealant or cellulosic acid (15, 19).

Patients treated with oral anticoagulants should not receive NSAIDs or paracetamol as analgesics during the perioperative period due to increased risk of hemorrhage while taking both types of medications simultaneously (1, 20). The first line analgesics in those patients are ibuprofen, naproxen or tramadol (15). INR testing should be made a day before the procedure and the value must be within the reference range. With INR > 3.5 the anticoagulant dose should be adjusted and 1 mg of vitamin K should be administered orally before the procedure or on the day of the procedure (1, 19). In case of procedures with higher hemorrhage risk (multiple extractions, periodontal procedures) introduction of low-molecular-weight heparin is required several days before and after the procedure.

This management algorithm generates numerous problems for the patients, who are often severely ill, as it requires multiple blood taking to perform a range of necessary tests. This management is even more burdensome in case of a patient reporting toothache and taking anticoagulants at the same time. It limits the time frame and urges the doctor to perform the procedure as soon as possible.

Although dental surgery procedures were qualified to the group of low post-operational hemorrhage risk (3, 13, 14), this possibility must always be considered. Usually a visit at the dentist's surgery involves stress and anxiety that may increase blood pressure temporarily, which, in combination with received medications, may evoke hemorrhage. In this case, bone wax constitutes a very good protection as it mechanically blocks vessel openings, in a similar way to a tampon. In order to minimize the

risk of hemorrhage, it is advisable to perform single tooth extractions.

Apart from numerous advantages of bone wax, available literature provides many examples of complications related to its use. Already in the 1950s, Geary and Frantz observed hindered healing of bone fractures while using wax in dogs (21). Bone wax may also penetrate into the lungs and cause embolism (22).

Excessive pressure resulting from wax application during neurological procedures of the spinal cord may lead to chronic pain (23) and even irritation of peripheral nerve function (24) or granuloma (25–27). Katre et al. described inferior alveolar nerve paralysis as complication after extraction of the wisdom tooth (28). Such a reaction was not found in this study but in view of anatomic proximity of the inferior alveolar nerve and the roots of the mandibular third molar, special care should be taken while using wax to reduce postoperative bleeding.

The most common complication while using this type of hemostatic material in surgery is inflammation (23, 29, 30). Leaving the wax longer than it is required for covering the wound with epithelium may result in irritation of the surrounding tissues, which was observed in this study. To avoid this condition, the wax should be precisely removed following 5–7 days of extraction without the risk of hemorrhage from the wound.

CONCLUSIONS

This study implies that bone wax is a good, efficient, and safe material to block bleeding from the alveolus following tooth extractions. Its efficacy was confirmed also in the group of patients receiving anticoagulants, eliminating the necessity of temporary withdrawal or adjustment of the anticoagulant therapy before the elective tooth extraction.

Due to possible allergic reactions to beeswax it is necessary to take medical history considering that issue before the procedure is done. As the material is not absorbed, it is important to instruct the patient about the necessity to remove residue wax from the alveolus after several days of the procedure or to schedule a follow-up visit to remove the wax. If the wax was left behind, local inflammation may occur caused by irritation of a foreign matter irritating the healing tissues.

Apart from hemostatic properties of the wax, authors of this article observed decreased tissue edema resulting from limited exudate as well as prevention of alveolar osteitis after using the described material. However, these observations need further investigation.

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GENERAL

**SAFETY OF ORAL IBUPROFEN – ANALYSIS OF DATA
FROM THE SPONTANEOUS REPORTING SYSTEM IN POLAND****ERNEST KUCHAR^{1*}, STANISŁAW HAN², KATARZYNA KARŁOWICZ-BODALSKA²,
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Abstract: Ibuprofen is a popular over-the-counter, non-steroidal anti-inflammatory medication, frequently used for the relief of fever, headaches, menstrual and other minor pains as well as a major active ingredient in numerous cold preparations. We analyzed sales volume and data obtained from the monitoring of spontaneous reports on the adverse effects of IBUM soft capsules, IBUM Forte soft capsules, and IBUM oral suspension 100 mg/5 mL collected by the manufacturer (PPF HASCO-LEK S.A. Wrocław, Poland) and National Monitoring Center in Warszawa in the period between October 2002 and June 2012. A total of 19,644,797 units of IBUM soft capsules 200 mg, 5,678,164 units of IBUM Forte soft capsules 400 mg and 4,333,325 units of IBUM oral suspension 100 mg/5 mL (29,656,286 units altogether) produced by PPF HASCO-LEK S.A. Wrocław, Poland were marketed during the period analyzed. There were 5 spontaneous reports regarding these medications registered in Poland in the period analyzed. Forms of oral ibuprofen are very safe medication rarely causing adverse effects; nevertheless, the existing spontaneous monitoring system of adverse effects in Poland is not sensitive enough to detect all adverse effects and needs improvement.

Keywords: pharmacoepidemiology, drug safety, pharmacovigilance, NSAID, adverse event

Abbreviations: COX – cyclooxygenase, FDA – Food and Drug Administration, NSAID – non-steroidal anti-inflammatory drug, OTC – over-the-counter

Ibuprofen, a propionic acid derivate, is a non-steroidal anti-inflammatory medication (NSAID) frequently used for the relief of fever, headaches, menstrual and other minor pains.

Patented in 1966, it was launched as a treatment in Great Britain by Boots Group in 1969 as Brufen (1). The patent's expiration in 1985 brought many generic drugs to the market.

At present, ibuprofen is one of the most widely used analgesics, anti-pyretics and anti-inflammatory drugs. It probably ranks after aspirin and paracetamol on the non-prescription over-the-counter (OTC) sale, although the patterns of use of these analgesics vary considerably from country to country (2).

Ibuprofen is a non-selective cyclooxygenase enzyme (COX) inhibitor, which inhibits COX-1 iso-

form responsible for physiologic action and COX-2 isoenzyme stimulated by the inflammation process. The COX enzyme catalyses the conversion of arachidonic acid to messenger molecules in the inflammation process: prostaglandins and thromboxane (2).

The most common adverse effects of oral ibuprofen are: dizziness, epigastric pain and heartburn. Adverse reactions such as nausea, vomiting, diarrhoea, flatulence, constipation, dyspepsia, abdominal pain, melaena, hematemesis, ulcerative stomatitis, gastritis exacerbation of colitis and Crohn's disease have also been reported following ibuprofen administration. Adverse effects such as pancreatitis, hypersensitivity reactions and various type of rashes have also been reported very rarely. Data from epidemiological sources and from a large

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number of controlled clinical trials performed in comparison with the newer coxib class of NSAIDs has shown that ibuprofen is amongst the NSAIDs with the lowest risk of serious gastrointestinal events (i.e., peptic ulcer bleeds etc.) (3).

Post-marketing surveillance using tools such as the data mining of spontaneous (passive) reports and the investigation of case reports to identify adverse drug reactions are very important in drug safety monitoring. Spontaneous reporting of suspected Adverse Drug Reactions (ADR) is well established as the most effective method of discovering possible new adverse reactions after medicinal products have received market approval.

PPF HASCO-LEK S.A. manufactures medicinal products with ibuprofen in the form of soft capsules (IBUM soft capsules 200 mg, IBUM Forte soft capsules 400 mg) as well as oral suspension (IBUM oral suspension 100 mg/5 mL). IBUM medicinal products have been on the Polish pharmaceutical market since October 2002. The manufacturer, as the Marketing Authorization Holder (MAH), has a pharmacovigilance system at his disposal, which is based on current EU legislation. As part of the pharmacovigilance system, the MAH monitors spontaneous reporting i.e., adverse reaction reports submitted by healthcare professionals and patients or their carers and world literature. PPF HASCO-LEK S.A. works closely with the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products based in Warszawa, which is the National Monitoring Centre in Poland. For new drugs, the monitoring actions aim to detect adverse reactions and interactions as well as groups of patients with a more frequent occurrence of adverse reactions.

Since no amount of pre-clinical and clinical data is sufficient to conclude the complete safety of a drug, it is necessary to report any adverse reaction of any pharmaceutical product to assess its safety to ensure patient health, which is the rationale of our study.

The identification of new, unknown adverse effects of oral ibuprofen and analysis of the safety of oral ibuprofen marketed by HASCO-LEK (IBUM soft capsules 200 mg, IBUM Forte soft capsules 400 mg, and IBUM oral suspension 100 mg/5 mL).

MATERIALS AND METHODS

We analyzed data obtained from the monitoring of spontaneous reports on adverse effects of IBUM soft capsules, IBUM Forte soft capsules, and IBUM oral suspension 100 mg/5 mL collected by

the manufacturer (PPF HASCO-LEK S.A. Wrocław, Poland) and National Monitoring Centre in Warszawa and sales volumes in the period from October 2002 to June 2012.

RESULTS

A total of 19,644,797 units of IBUM soft capsules 200 mg, 5,678,164 units of IBUM Forte soft capsules 400 mg and 4,333,325 units of IBUM oral suspension 100 mg/5 mL (29,656,286 units altogether) produced by PPF HASCO-LEK S.A. Wrocław, Poland were marketed during the period analyzed.

There were 5 spontaneous reports regarding medications registered in Poland in the period analyzed.

1. An adult male reported a heavy burning sensation in his mouth as well as edema and irritation of the throat after chewing the IBUM Forte capsule, which is not recommended by the manufacturer. The symptoms subsided within about 20 min after gargling with water.
2. A 38-year old male complained of a burning sensation on his tongue after he tasted the IBUM oral suspension to check its taste.
3. An adult female complained of a burning sensation on her tongue after she tasted the IBUM oral suspension to check its taste.

The two above mentioned reports were received shortly after the manufacturer changed the color of the IBUM oral suspension from pink to white, which triggered anxiety in parents of child consumers.

4. A 6-year old girl weighing 24 kg developed generalized itching and a rash, prominently on her buttocks, knees and elbow folds after she had been administered IBUM oral suspension at a daily dose of 30 mL (i.e., 600 mg; 25 mg/kg) for 4 consecutive days. The girl was found to be allergic to the drug.
5. A 4-year old boy weighing 11 kg, developed generalized urticaria (hives) shortly after a single 5 mL dose of the IBUM suspension given for fever. The boy was found to be oversensitive to NSAIDs.

DISCUSSION

Following the thalidomide tragedy in 1961, the WHO pursued measures aiming at the prompt detection and publication of information relating to unknown and poorly understood adverse effects, which, in 1978, contributed to the creation of a

worldwide pharmacovigilance network that now includes more than 130 countries, known as the WHO Programme for International Drug Monitoring with its headquarters in The Uppsala Monitoring Centre, Uppsala, Sweden (4). Therefore, in most countries worldwide, drug-related adverse reactions are monitored in order to increase the safety of pharmacological therapy. A new field of science, called pharmacovigilance, came into being (5). As part of clinical trials preceding the launch of a drug, the most frequent adverse reactions are detected. Due to the number of subjects, very rare adverse effects with a frequency lower than 1 : 10,000 are hardly detectable. Antihistamine drugs used to treat common allergies are ubiquitous remedies administered in millions of patients worldwide annually. With such extensive drug use, extremely rare adverse events, even those < 1 per million, are unavoidable. Detecting these rare events is the issue to be addressed in the studies. Due to practical and financial reasons, active surveillance of millions of patients is not viable, thus pharmacovigilance is based on the analysis of spontaneous reports from healthcare professionals and consumers. The assessment of the frequency of rare adverse events is based on an equation with a numerator – the number of events – and a denominator – the number of therapies. The problem is that not all adverse reactions are reported, which results in decreased sensitivity and difficulties assessing the number of treatments. In practice, the surrogate for the number of therapies is the number of drug packs available on the market. Another issue is the difficulty establishing drug use causality (effect or coincidence), especially in the case of concomitant drug use as discussed in the report analyzed herein. In general, a causal relationship depends upon the nature and amount of evidence supporting an attribution hypothesis, such that “medication X causes adverse effect Y”. X can be a sufficient cause of Y, meaning that X is always followed by Y; or a necessary cause, meaning that Y cannot occur without being preceded by X; or both. These deterministic concepts are not relevant to pharmacovigilance. No drug is a sufficient cause of any adverse effect, and there are no examples of necessary causes either. The trigger for modern pharmacovigilance, thalidomide, has come close to being considered a necessary cause of a congenital defect, phocomelia, but this is not true. Phocomelia is very rare in the absence of thalidomide but does happen; x-irradiation is one example of another cause (6).

Pharmacovigilance in action is essentially Bayesian: a tentative prior probability for a hypothesis becomes modified up or down as further evi-

dence is obtained, to become a posterior probability for new consideration. Such evidence may come from different sources and also the overall view of the data may use different logic. Individual case reports can provide excellent evidence on attribution in one or more specific patients (6)

Among the reported cases, the first one occurred due to medical error connected with the incorrect administration of IBUM Forte soft capsules 400 mg. A soft capsule is a pharmaceutical form, which encloses medicines in a relatively stable shell allowing them to be taken orally. It should be swallowed not chewed by patients. Irritation connected with the drug crushed in the mouth can be connected with a relatively high concentration of the active substance or other ingredient, but it can also be a hypersensitivity reaction to one of the ingredients. Two registered reports from suspicious parents of a burning sensation on the tongue after tasting IBUM oral suspension were received shortly after the manufacturer changed the color of the drug from red to white (a colorant had been removed). The color change could have triggered anxiety in parents, who reported their own taste sensation.

Two reported adverse reactions connected with skin and soft tissue disorders were assessed as hypersensitivity reactions due to ibuprofen use. These cases were non-serious reactions.

Assessment of the occurrence probability of the reported reactions described above after ibuprofen administration shows ibuprofen as a possible cause according to WHO criteria (4).

Ibuprofen rarely causes skin-related adverse reactions, especially fixed drug eruptions (7). However, in the literature there were described either general drug eruption like in one reported case or urticaria in another one. Shelley and Shelley (8) described 4 cases of ibuprofen urticaria, which have been observed in 3 adults and a 14-year old girl. Diaz Jara et al. (9) reported urticaria after 120 mg of ibuprofen administration to 7-year old boy in the treatment of a headache.

According to data from clinical trials, ibuprofen is believed to have a very good clinical safety profile. In large randomized controlled clinical trials in which 83,915 children participated, adverse events were only observed in 79 children among 54,785 treated with ibuprofen. The following was noted: abdominal pain in 4 children, asthma in 44 children, multiforme erythema in 3 children, leucopenia in 8 and gastritis in 20 (10). FDA acknowledge ibuprofen as a very safe NSAID (11).

In the 18-year period, a total of 5,042 adverse event reports were received for ibuprofen from the

USA. Of these, 768 (15.2%) were serious. Clinically significant reports, such as GI bleeding and perforation (71 cases) were infrequent over the 18-year reporting period for the US population, which is estimated at 300 million. Of the 324 serious GI reports received during this 18-year period, death was noted in four, presumably due to GI bleeding. Two decades of post-marketing experience in the United States have established that the reporting frequency of serious GI events remained consistently low during this time, suggesting that ibuprofen, which is available over-the-counter, is well tolerated in the general population (11).

Analyzing all safety signals reported in nearly 10-years of marketing of IBUM drugs in Poland allows us to estimate that oral ibuprofen is a safe medication. But only 5 cases of adverse reactions reported against almost 30 million treatments might be a signal of the low sensitivity of the existing monitoring system. According to the FDA data transferred into the Polish population, few reports of adverse reactions are expected annually. Similarly, in the RxISK electronic database (<https://www.rxisk.org/Default.aspx>) only 20,590 reports were found where ibuprofen was the suspected drug, covering 78,271 reactions. The main reason for the small number of reports received might be due to medical practitioners' poor awareness of the importance of all safety signals, including all well-known and common adverse reactions.

The weakness of this paper is that it is based on passive adverse event reports and its strength is almost 10- years of observation of the entire Polish population.

Forms of oral ibuprofen are very safe rarely causing adverse reactions. The scarce number of safety reports shows that the existing spontaneous monitoring system in Poland seems not to be sensitive enough to detect all adverse effects and needs improvement.

Conflicts of interest

Ernest Kuchar has received funding or honoraria for lectures, conference attendance, or consultancy from a number of pharmaceutical companies including PPF HASCO-LEK S.A. Stanisław Han is the President of the Management Board of PPF HASCO-LEK S.A. Katarzyna Karłowicz-Bodalska is a Research Director in PPF HASCO-LEK S.A. Elżbieta Kuttycka is a Pharmacovigilance Department Manager in PPF HASCO-LEK S.A.

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SHORT COMMUNICATION

THE PENETRATION OF SUNITINIB THROUGH THE BLOOD-BRAIN BARRIER AFTER THE ADMINISTRATION OF CIPROFLOXACIN

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and EDMUND GRZEŚKOWIAK¹¹Department of Clinical Pharmacy and Biopharmacy, ²Department of Teaching Anaesthesiology and Intensive Therapy, Poznan University of Medical Sciences, Św. Marii Magdaleny 14, 61-861 Poznań, Poland³ Polpharma Biologics, Trzy lipy 3, 80-172 Gdańsk, Poland**Keywords:** sunitinib, blood-brain barrier, ciprofloxacin, P-gp, rabbits

Sunitinib (SU 11248) is an efficacious antineoplastic and antiangiogenic drug from the group of tyrosine kinase inhibitors (TKIs). In 2006, SUTENT[®] (Pfizer) was registered by the US Food and Drug Administration (FDA) for the treatment of gastrointestinal stromal tumor (GIST) if the imatinib therapy is unsuccessful and for the treatment of renal cell carcinoma (RCC) (1). In 2011, SUTENT[®] was recommended by the FDA for the treatment of progressive pancreatic neuroendocrine tumors (2). Apart from the indications listed above, the drug may also be efficacious in non-small-cell lung cancer, breast cancer and neuroendocrine neoplasms (3). The drug achieves its antineoplastic effect by inhibition of the following receptors: platelet-derived growth factor receptors (PDGFRa and PDGFRb), vascular endothelial growth factor receptors (VEGFR1, VEGFR2, VEGFR3), stem cell factor receptor (KIT), Fms-like tyrosine kinase receptor-3 (FLT3), colony stimulating factor receptor (CSF-1R), glial cell line-derived neurotrophic factor receptor (RET). The pharmacokinetics of sunitinib was studied in a number of investigations. The bioavailability of the drug in animals is about 50% (4). The maximum concentration of the drug in the plasma (C_{max}) is observed between 6 and 12 h after the administration (5). A daily dose of 50 mg of sunitinib gives a possibility to reach the steady-state

concentration of 50–100 ng/mL, which influences the pharmacological effect of the drug (6). The time necessary to achieve the steady state is 7–14 days for sunitinib and about 14 days for its active metabolite SU 12662 (N-desethylsunitinib). The administration of the drug once a day for 14 days causes the concentration of sunitinib to increase 4.5 times; the concentration of the active metabolite increases 10 times and the complete fraction of the drug (sunitinib plus SU 12662) increases 5 times (7). CYP3A4 has been proved to be involved in the metabolism of sunitinib and its major circulating metabolite (SU 12662) (5, 6, 8), which unfortunately may be the cause of interaction with the inhibitors or inductors of this enzyme when they are simultaneously applied (9).

At present, there are only a few publications available, which support the opinion that sunitinib may cross the blood-brain barrier. However, few authors confirm sunitinib activity in brain metastases from kidney cancer (10–13). Patyna et al. (14) showed rapid distribution of [¹⁴C]-sunitinib and its metabolite in the brain and spinal cord tissue after intravenous or oral administration of the drug to monkeys, rats and mice. The conclusion of the research was the need to continue it due to the anti-tumor activity in the brain. There is growing interest in the subject because of the efficaciousness of sunitinib

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tinib in the group of patients with brain metastases from kidney cancer. P-glycoprotein (P-gp; ABCB1) plays a significant role in the transport of drugs through the blood-brain barrier (15–17). Numerous data demonstrated that P-gp inhibition mediated by some drugs could significantly increase brain fluid concentrations by increasing its brain permeability (18, 19). Sunitinib and SU12662 are the substrates of ABCB1 (20, 21). Increased permeability of sunitinib to the central nervous system (CNS) was observed in mice void of ABCB1 protein (20). Tang et al. proved higher accumulation of sunitinib in the brain when it was administered with elacridar, P-gp inhibitor. However, this effect was not proved for SU12662 (22).

Ciprofloxacin is used in the treatment of infections caused especially by most Gram-negative pathogens. It is still highly effective against *Pseudomonas aeruginosa*. The antibiotic is less active against Gram-positive pathogens (23, 24). Because of its good tissue penetration the fluoroquinolone is efficacious in the treatment of urinary tract infections, skin and bone infections, gastrointestinal infections, lower respiratory tract infections, febrile neutropenia, intraabdominal infections (23, 25, 26). Ciprofloxacin is an inhibitor of cytochrome P450 CYP3A4 and causes numerous drug interactions (27–30). The authors proved a significant increase in the area under the curve (AUC) and C_{max} of sunitinib after the administration of the TKI with ciprofloxacin to rabbits (31). The literature does not provide unequivocal data concerning the influence of ciprofloxacin on P-gp (32). Such fluoroquinolones as grepafloxacin, levofloxacin and sparfloxacin inhibited the efflux of erythromycin *in vitro* (33). deLange et al. proved the inhibition of ciprofloxacin on the transport of rhodamine, a P-gp substrate (34).

The current study was conducted to investigate the inhibitory effect of ciprofloxacin, a widely used fluoroquinolone, on the penetration of sunitinib into the cerebrospinal fluid. It is thought that the inhibition of P-gp at the blood-brain barrier may increase the cerebrospinal penetration of sunitinib and in consequence increase its effectiveness in the treatment of brain metastases from kidney cancer. Due to the fact that there is high probability of combination of both drugs in patients with renal cancer, ciprofloxacin was used in the research.

We searched the bibliographic database of the National Library of Medicine (MEDLINE®) and found no evidence of the effect in the literature.

EXPERIMENTAL

Reagents

Sunitinib and SU12662 were purchased from LGC Standards (Łomianki, Poland), HPLC grade acetonitrile, ammonium acetate and acetic acid from Sigma-Aldrich and methanol from Merck. Water used in the mobile phase was deionized, distilled and filtered through a Millipore system before use. SUTENT® was purchased (batch number P177H) from Pfizer Trading Polska Sp. z o.o., Warszawa, Poland.

Animals

Adult New Zealand male rabbits, weighing 2.5–3.4 kg, were used for experiments. All the rabbits were kept in individual metal cages located in the animal laboratory of the Poznań University of Medical Sciences, Department and Unit of Clinical Pharmacy and Biopharmacy. They were acclimatized for two weeks prior to the experiments and were maintained under standard conditions of temperature ($23 \pm 2^\circ\text{C}$) and humidity (56–60%) with an alternating 12 h light/dark cycles. New Zealand rabbits were provided with 100 g of commercial pelleted diet (Labofeed KB®: 9.8 MJ/kg metabolic energy, 16.00% total protein, 0.65% vitamin P, 15,000 IU vitamin A, 1500 IU vitamin D₃, and 65 mg vitamin E) and tap water *ad libitum*. All experimental procedures related to this study were approved by the Local Ethics Committee of Poznań University of Medical Sciences.

Evaluation of sunitinib and SU12662 pharmacokinetics

The rabbits were divided into two groups (7 animals in each): I (control) – receiving sunitinib, II – receiving sunitinib and ciprofloxacin. Ciprofloxacin (Proxacin® 10 mg/mL; Polfa, Warszawa, Poland) as solution was administered over 30 min intravenously with an infusion pump through the left marginal ear vein at the doses of 20 mg/kg/12 h (35). The steady-state was achieved by multiple administration of ciprofloxacin. After administration of the 7th dose of the antibiotic, sunitinib (SUTENT® 25 mg) was administered *per os* (*p.o.*) at the single dose of 25 mg (36). The serum and CSF samples were taken from one rabbit at the particular time points. The rabbits were anesthetized with intramuscular ketamine (30 mg/kg) and xylazine (4 mg/kg) into thigh. Ten minutes later, after the onset of anesthesia, the animals were placed in a lateral recumbent position in order to maintain the patent airways. The rabbits' respiratory

movements were continuously observed to detect the symptoms of respiratory insufficiency. During the anesthesia, an 18-G catheter was inserted into the central auricular artery for blood sampling. The hair above the neck skin was removed and the skin surface was disinfected. In the lateral recumbent position, the head was flexed and the landmarks for needle placement (the occipital protuberance and the cranial margins of the wings of the atlas) were palpated. A 20G (0.9 × 40 mm) sterile needle (Terumo Europe N.V.) needle was inserted gently in the mid-line at 90 degrees to the vertebral column, and 0.5 mL of the cerebrospinal fluid (CSF) was collected into a plastic container (SafeSeal microtube, Sarstedt). No syringe aspiration was applied. After CSF sampling, the animals were euthanized with intravenous sodium pentobarbital (Morbital, Vetoquinol-Biowet) 1 mL/kg administered into the marginal ear vein.

Blood (3 mL) and CSF (100 µL) samples for sunitinib, SU12662 assays were collected before and 2, 4, 6, 8, 12, 24 h following sunitinib administration. The blood samples were transferred into heparinized tubes and they were centrifuged at 4000 rpm for 8 min at 4°C. Next, the plasma was transferred to propylene tubes and stored at -20°C until analysis.

The measurement of sunitinib concentration in the blood plasma and CSF was made by means of the HPLC (high-performance liquid chromatography) method with UV detection, which was a modi-

fication of the method developed by Faivre et al. (37). Separation was achieved by isocratic elution of the mobile phase, ammonium acetate 20 mM pH 3.4 (adjusted with acetic acid) – acetonitrile (60 : 40, v/v), at a flow rate of 1.0 mL/min through a Symmetry® RP-C8 column (250 mm × 4.6 mm, 5.0 mm particle size) (Waters®). The column temperature was maintained at 40°C, the UV detection wavelength was set at 431 nm. The total analysis time for each run was 6 min. The lower limit of quantification (LLOQ) and limit of detection (LOD) for sunitinib and SU12662 were 1.0 ng/mL and 0.5 ng/mL, respectively. Intra- and inter-day precision and accuracy of the low quality control (2.5 ng/mL), medium quality control (25.0, 125.0 ng/mL), and high quality control (45.0, 200.0 ng/mL) were well within the acceptable limit of 10% coefficient of variation (CV%) for SU12662 and sunitinib, respectively. The calibration for sunitinib was linear in the range 1.0–250.0 ng/mL ($r = 0.999$), for SU12662 in the range 1.0–50.0 ng/mL ($r = 0.998$).

Pharmacokinetics analysis

Pharmacokinetic parameters for sunitinib and its metabolite were estimated by non-compartmental methods using validated software (Phoenix™ WinNonlin® 6.3; Certara L.P., USA). The following pharmacokinetic parameters were calculated for sunitinib in the blood and cerebrospinal fluid: maximum observed plasma concentration (C_{max}); time to reach maximum concentration (t_{max}); area under the

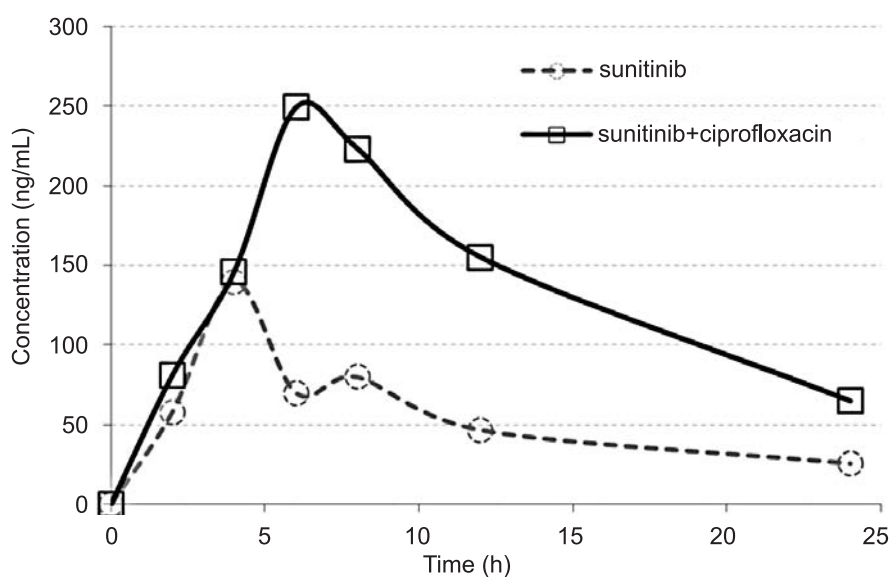


Figure 1. Sunitinib plasma concentration–time profiles following a single oral dose of sunitinib 25 mg in rabbits

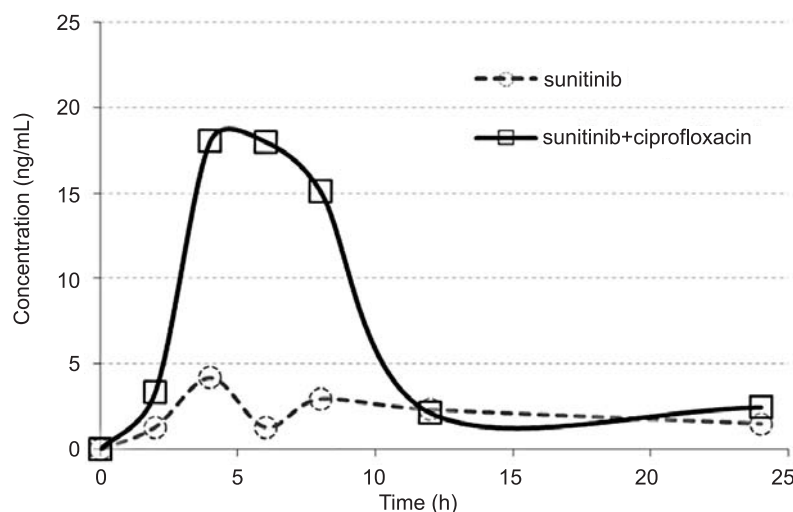


Figure 2. Sunitinib cerebrospinal concentration–time profiles following a single oral dose of sunitinib 25 mg in rabbits

plasma concentration–time curve from zero to 12 h ($AUC_{0-12\text{ h}}$); area under the plasma concentration–time curve from zero to 24 h ($AUC_{0-24\text{ h}}$), area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$), half-life in elimination phase ($t_{1/2\text{el}}$), clearance (CL), area under the first moment curve ($AUMC_{0-24\text{ h}}$), mean residence time (MRT).

RESULTS

The two analyzed groups under study did not differ significantly in body mass.

Plasma C_{max} for sunitinib in the control and sunitinib + ciprofloxacin groups were 139.5 and 248.9 ng/mL, respectively (Table 1). Cerebrospinal C_{max} for sunitinib in the control and sunitinib + ciprofloxacin groups were 4.2 and 18.0 ng/mL, respectively. Plasma $AUC_{0-24\text{ h}}$ for sunitinib in the control and sunitinib + ciprofloxacin groups were 1304.6 and 3254.2 ng \times h/mL, respectively. Cerebrospinal $AUC_{0-24\text{ h}}$ for sunitinib in the control and sunitinib + ciprofloxacin groups were 50.4 and 155.9 ng \times h/mL, respectively (Table 1). All calculated pharmacokinetic parameters for both groups are presented in Table 1. There was no measurable levels of metabolite in CSF.

The coefficient of drug penetration through the blood–brain barrier was estimated from the ratio of the sunitinib concentration in CSF over the plasma concentration ($C_{\text{CSF}}/C_{\text{plasma}}$). The coefficients of sunitinib penetration through the blood–brain barrier are not quite similar (range 0.019–0.059 vs. 0.014–0.123 for group I and II, respectively).

The concentrations of sunitinib in the blood plasma for the two groups are shown in Figure 1. The concentrations of sunitinib in the cerebrospinal fluid in analyzed rabbits are shown in Figure 2.

DISCUSSION

The blood–brain barrier is developed to maintain and regulate the microenvironment of the CNS and is composed of the endothelial tight junctions, which are distant to the epithelial ones. The molecular components include the set of proteins: claudins, occludin, ZO-1, 2, 3, cingulin and 7H6 creating the barrier and being responsible for the communication with the external environment using G-proteins, serine-, threonine- and tyrosine-kinases, calcium levels, cAMP, proteases and cytokines (38). Astrocytes contact the subendothelial basal lamina creating orthogonal arrays of particles (OAPs) containing the water channel protein aquaporin-4 mediating the water movement between the compartments. The blood–brain barrier is extensively disturbed in area of the brain tumors (e.g., glioblastomas, astrocytomas) thus allowing some drugs, normally not able to reach the main bulk of gliomas, to cross the barrier. P-gp is an important component of the blood–brain barrier. Blood capillaries are rich in ABCB1, and the protein takes part in the transport of numerous substances. It exhibits affinity for a broad spectrum of lipophilic substances, which are not structurally related with each other (e.g., digoxin, cyclosporin A, HIV protease inhibitors) (39–41). On the one hand, its effect is reduced exposure of

Table 1. Plasma and CSF pharmacokinetic parameters for sunitinib and metabolite (SU12662) following a single oral dose of sunitinib 25 mg.

Pharmacokinetics parameters ^a	I			II		
	CSF	plasma	CSF/plasma ratio	CSF	plasma	CSF/plasma ratio
Sunitinib						
AUC _{0-12h} (ng×h/mL)	27.2	869.2	0.031	128.2	1932.6	0.066
AUC _{0-24h} (ng×h/mL)	50.4	1304.6	0.039	155.9	3254.2	0.048
AUC _{0-∞} (ng×h/mL)	88.0	1698.0	0.052	185.4	4111.1	0.045
C _{max} (ng/mL)	4.2	139.5	0.030	18.0	248.9	0.072
t _{max} (h)	4.0	4.0	1.0	4.0	6.0	0.667
MRT (h)	11.3	9.4	1.20	8.3	10.5	0.762
AUMC _{0-24h} (ng×h ² /mL)	570.1	12317.4	0.046	1297.0	34106.7	0.038
SU12662						
AUC _{0-24h} (ng×h/mL)	-	212.3	-	-	258.2	-
AUC _{0-∞} (ng×h/mL)	-	274.6	-	-	311.1	-
C _{max} (ng/mL)	-	12.4	-	-	33.3	-
t _{max} (h)	-	8	-	-	8	-
AUMC _{0-24h} (ng×h ² /mL)	-	2362.7	-	-	2611.4	-

I, sunitinib; II, sunitinib + ciprofloxacin; AUC_{0-12h} – area under the plasma concentration-time curve from zero to 12h; AUC_{0-24h} – area under the plasma concentration-time curve from zero to 24h; AUC_{0-∞} – area under the plasma concentration-time curve from zero to infinity; C_{max} – maximum observed plasma concentration; t_{max} – time to reach maximum concentration; MRT – mean residence time; AUMC_{0-24h} – area under the first moment curve; CSF, cerebrospinal fluid

the brain to toxic compounds. However, on the other hand, the activity of P-gp may lead to lower effectiveness of the treatment of brain tumors, for example. Thus, it seems logical that the application of a combined therapy with a P-gp inhibitor should result in better availability of the antineoplastic drug in the brain and improved clinical efficacy in diseases of the CNS. The aim of the research was to determine the influence of ciprofloxacin on the penetration of sunitinib into the CSF. The influence of ciprofloxacin on P-gp has not been clearly determined. Park et al. explain that the application of different cell lines with P-gp overexpression may lead to different, usually contradictory, conclusions whether or not a specific drug is a substrate of P-gp. In his research Park concludes that some cell lines (MDCKI and MDCKI-MDR1) indicate that ciprofloxacin is a substrate of P-gp, but data from other cells (MDCKII, MDCKII-MDR1, LLC-PK1 and L-MDR1) contradict it. Brain accumulation of sunitinib and its metabolite is restricted by ABCB1 (23). Additionally, sunitinib inhibits the function of the ATP-Binding Cassette (ABC) transporter P-gp (42), which may have influence on the availability of simultaneously applied drugs (42).

In the group of animals with ciprofloxacin the C_{max} of sunitinib in the CSF was 329% higher than

in the control group, whereas AUC_{0-24h} was 209% higher, which resulted from the higher parameters of C_{max} and AUC of sunitinib in the blood in the group with the antibiotic (Table 1). Higher plasma concentrations of active metabolite SU12662 correspond with higher plasma levels of sunitinib. There was no measurable levels of metabolite in CSF. The obtained values of the pharmacokinetic parameters in the plasma for sunitinib confirm the results obtained by the authors in their earlier research on the influence of fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin) on the pharmacokinetics of sunitinib (31), where values of mean maximum TKI concentration in the sunitinib + ciprofloxacin group and in the control group were 203.8 ± 50.6 vs. 111.8 ± 20.9 ng/mL, respectively. The results point to the inhibiting effect of ciprofloxacin on CYP3A4.

In view of the fact that in both analyzed groups the coefficients of sunitinib penetration through the blood-brain barrier are very low we can suppose that the influence of ciprofloxacin on P-gp was not significant.

The research was limited by a small number of animals. Nevertheless, even with this number of animals we succeeded in proving visible differences in the PK parameters between the groups. The higher con-

centration of sunitinib in the CSF is the consequence of higher TKI concentration in the plasma, which probably results from the inhibiting effect of ciprofloxacin on the metabolism of sunitinib. The values of the coefficients of TKI penetration through the blood-brain barrier in both groups seem to indicate that the antibiotic has little influence on P-glycoprotein.

CONCLUSION

The study revealed increased concentrations of sunitinib in the cerebrospinal fluid after the administration of ciprofloxacin, but probably it was an effect of the inhibition of CYP3A4 by the antibiotic and increased sunitinib concentrations in the plasma rather than its influence on P-gp.

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