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CONTENTS

REVIEW

557. Fei Wang, Yang Wang, Ningning Hu, Xuman Miao
Risks factors, pathogenesis and pharmaceutical approaches for treatment of steroid-induced bone infarction of femoral head.

565. Muhammad Atif, Ikükar Ali, Ajaz Hassan, Syed Visar Hyder, Farhan Ahmed Khan, Aneela Maalik, Umair Farooq
Pharmacological assessment of hispidulin - a natural bioactive flavone.

579. Renata Francik, Jadwiga Kryczyk, Sławomir Francik
Coronary effect of fibrates on proteins and enzymes which hydrolyze triacylglycerols.

589. Bożena Muszyńska, Katarzyna Kała, Anna Firlej, Katarzyna Sulowska-Ziaja
Cantharellus cibarius – culinary-medicinal mushroom content and biological activity.

599. Paweł Petryszyn, Anna Wielą-Hojeńska
Economic issues in therapeutic drug monitoring.

605. Maciej Bilek, Jacek Namieśnik
Chromatographic techniques in pharmaceutical analysis in Poland: history and the presence on the basis of papers published in selected Polish pharmaceutical journals in XX century.

ANALYSIS

613. Paulius Ciegis, Andrejus Zevzikovas, Augusta Zevzikoviene, Palma Nenortiene, Daiva Kazlauskienė
Investigation of a mixture containing alprazolam, codeine, and paracetamol using thin-layer and high performance liquid chromatography methods.

621. Piotr Garbacki, Judyta Cielecka-Piонтek, Przemysław Zelewski, Irena Oszczapowicz, Anna Jelińska
A simple and sensitive stability-indicating UHPLC-DAD method for determination of cefetamet pivoxil hydrochloride.

627. Kudige Nagaraj Prashanth, Nagaraju Swamy, Kanakapura Basavaiah
Rapid spectrophotometric determination of trifluoperazine dihydrochloride as base form in pharmaceutical formulation through charge-transfer complexation.

DRUG BIOCHEMISTRY

637. Anna Majewska, Witold Lasek, Michał Janyst, Grażyna Mlynarczyk
In vitro inhibition of HHV-1 replication by inosine pranobex and interferon-α.

645. Adam Guśpiel, Joanna Ziemska, Agnieszka Czopič, Robert Kawički, Jolanta Solecka
Intracellular antioxidant activity of a Streptomyces sp. 8812 secondary metabolite, 6,7-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid and its synthetic derivatives.

653. Dorota Wreznow, Martyna Oprzonk, Anna Hechmann, Artur Beberok, Michał Otryba, Ewa Bussman
Effect of paracetamol on melanization process in human epidermal melanocytes.

659. Azar Hosseini, Davood Mahdian
Protective effect of Lactuca serriola on doxorubicin-induced toxicity in H9C2 cells.

667. Muhammad Hasham Hassan Bin Asad, Muhammad Iqbal, Muhammad Rosuf Akram, Naem Raza Khawaja, Saïqa Munir, Muhammad Zubair Shabbir, Muhammad Saqib Khan, Ghalam Murtaza, Izhar Hussain
5’-Nucleotidases of Naja naja karachensis snake venom: their determination, toxicities and remedial approach by natural inhibitors (medicinal plants).

675. Mohammed M. Salhi, Mohammad Firoz Alam, Gulrana Khuwaja, Farah Islam, Sohail Hussain, Mohnsen Mohammed Fageeh, Tariq Anwer, Fakhru Islam
Repeated exposure of sodium tellurite on the rat liver and on the potential mechanisms of the metalloid-induced hepatotoxicity.

DRUG SYNTHESIS

683. Katarzyna Badowska-Rosłonek, Agnieszka Ciesielska, Marta Świtalska, Małgorzata Piskozub, Wanda Peczyńska-Czoch, Joanna Wietrzyk, Łukasz Kuczmarek
693. Anna Bielenica, Joanna Stefąska, Anna E. Kozio≥, Thiourea derivatives of 4-azatricyclo[5.2.2.0 2,6]undec-8-ene-3,5- dione - synthesis and biological activity.

705. Anna Wińiewska, Piotr F.J. LipiΩski, Krzysztof Wozniak, Synthesis and antimicrobial properties of new mandelate ionic liquids.

**NATURAL DRUGS**

717. Alamgeer, Huma Naz, Shahid Rasool, Sayed Atif Raza, Anti-inflammatory, analgesic and antipyretic activities of the aqueous methanolic extract of *Berberis calliobotrys* in albino mice.

**PHARMACEUTICAL TECHNOLOGY**

725. Ramzi Shawahna, Abdel Kareem Hroub, Eliama Abed, Pharmaceutical quality of generic atorvastatin products compared with the innovator product: a need for revising pricing policy in Palestine.

731. Tomasz Stawarski, Edmund Sieradzki, Emilia Galecka, Karolina Binek, Dissolution and compatibility study of binary and ternary interactive mixtures of indomethacin: comparison with commercially available capsules.

739. Hamzah M. Maswadeh, Development and verification of new solid dental filling temporary materials containing zinc. Formula development stage.

**PHARMACOLOGY**

761. Ma≥gorzata Zygmunt, Graøyna Ch≥oÒ-Rzepa, Anti-inflammatory and antioxidant activity of 8-methoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl derivatives with terminal carboxylic, ester or amide moieties in animal models.


777. Łukasz Dobrek, Beata Skowron, Agnieszka Baranowska, The influence of montelukast on the activity of the autonomic nervous system estimated by heart rate variability in experimental partial bladder outlet obstruction in rats.

**GENERAL**


808. Erratum.
The progressive fragmentation of bones due to interrupted blood supply is called bone infarction (BI) (1, 2). Chronic use of steroids may produce fat emboli and thrombotic occlusion. It leads to reduction in blood supply to bones resulting in osteocytes necrosis or steroid induced bone infarction (SIBI). It is a painful condition, which resists to patient’s movement. The severity of pain increases with time. Various treatment or preventive options are available if diagnosed earlier; however, late diagnosis complicates the choice of therapeutic approaches. In advanced disease state, only option is surgery of necrotic region (3-9).

Hypercortisolism is the non-traumatic cause of BI. The femoral head is the most common location for SIBI. The incidence of BI is high in young and active individuals. Beside it, other skeletal parts including shoulder, knee, ankle and hand are also affected by SIBI (13). Hypercortisolism results from systemic administration of steroids in large doses for prolonged period i.e., up to 90 days or longer (12-22).

For example, methylprednisolone-induced femoral head osteonecrosis, however prediction of its development in a specific patient is not feasible (23). In a study on 6000 patients with femoral head injuries, no signs of osteonecrosis were observed after chronic use of high-dose steroids (24). In another study conducted on patients receiving high-dose steroids, the prevalence of osteonecrosis was not greater than five percent (23). Quite the opposite, the development of osteonecrosis has been reported in patients receiving high-dose steroids for short period (24). Some studies have also reported SIBI of the femoral head after comparatively short periods (one week) of oral steroids (13). First case of SIBI was reported in 1957, and number of reported cases increased to 154 in 1968, due to which SIBI is known as a disease of medical progress (19). However, the cautious use of steroids has now resulted in significant decrease in the incidence of BI among patients, chronically treated with systemic steroids (20). As far as epidemiology of glucocorticoid-induced BI is concerned, SIBI...
produces significant morbidity accounting for approximately 10% of all cases of total hip replacement (THR) in the United States (12). The incidence of SIBI with systemic lupus erythematosus and post-renal transplantation as co-morbidities is 3-41% (13) and 4-40% (1), respectively. This prevalence feature depends on the dosage of steroids and their route of administration.

**Mode of pathogenesis**

The exact pathology of BI is still undiscovered. Based on a hypothesis, BI is developed because of osteocyte necrosis and blood vessel blockage leading to reduced blood flow to bone, which results in BI. Mechanistically, osteocyte necrosis occurs due to apoptosis and fat cell hypertrophy (FCH), while fat emboli causes blood vessel blockage.

Wang et al. administered high-dose cortisone to rabbits and observed the production of FCH and fat embolism, which caused abolition of blood vessels in the subchondral bone of femur. In addition, they also noted the increased number of marrow fat cells in these rabbits (25). After administration of dexamethasone to pluripotent cell line acquired from mice bone marrow, Cui et al. also observed in vitro FCH and hypothesized of FCH-induced expansion of cell volume within a limited volume of the femoral head. It reduces the perfusion of blood leading to BI (26). Conversely, the complement pathway is activated by fat emboli dumped within the subchondral vessels and sinusoids. It causes the dumping of immune complex followed by the initiation of various thrombotic processes including intravascular coagulation and then the development of BI (27).

In a study to demonstrate the effect of lipid lowering agent (clofibrate) in the steroid-treated rabbits, the researchers observed the decreased fat cell size and intra-cortical pressure with subsequent improvement of blood flow (28-30).

Another pathologic factor for BI is the apoptosis of osteocytes in the affected bones (31, 32), which is not observed in BI induced by other factors including alcohol usage or trauma (33). Calder et al. and Weinstein et al. observed the apoptotic osteocytes in pathologic specimen of femoral head during THR in patients suffering from SIBI. Owing to gradual accumulation of these apoptotic osteocytes in the bony tissues, osteocyte-lacunar-canalicular system is disrupted and eventually femoral head collapse takes place (31-33).

**Steroid-induced bone infarction regions**

The literature survey showed various studies conducted on systemic lupus erythematosus (SLE) patients who were chronically treated with steroids (34-36). In these patients, the diagnostic findings revealed SIBI at multiple sites (more than two joint regions). Various case studies elaborated that the SLE patients with BI involving multiple joint regions ranged between 70-90% (34-36). One of these studies conducted on 95 SLE patients with symptomatic BI reported that hip (54.7%) and knee (18.9%) joints were the most frequently affected regions. Another study revealed 12% incidence of BI in SLE patients who received a considerably higher average daily dose of prednisolone (15.62 mg) in comparison to SLE control patients (9.3%) with no BI (5 mg) (8). The most affected site in these patients was the hip joint in 95% patients which was bilateral naturally in 72% of patients. Similar results were reported in another study that showed the incidence rate of SIBI by 82%, 64%, 20%, and 25% in hip, knee, ankle, and shoulder joints, respectively (37).

**Risk factors**

There are conflicts among different studies on the association between BI development and steroid usage factors, i.e., dose, route of administration and duration of use. Some studies report the emergence of BI after prolonged, high-dose steroids usage (15, 38, 39), while few investigators claim that high-dose steroids usage for short period may also produce BI (39-41). For example, BI was observed in significantly higher number of SLE patients who received methylprednisolone pulse treatment (19%) as compared to control group of SLE patients who did not receive methylprednisolone pulse treatment (6%) (40). In another study, BI was not noted in 17 SLE patients who received steroid pulse treatment (42). Moreover, some investigators have reported the development of BI after steroids usage through various routes including oral, intravenous, intra-articular and intramuscular (43, 44).

In some meta-analysis studies, positive relationship between BI development in SLE patients after renal transplantation and daily dose of steroids was determined (15, 45-52). A retrospective study revealed the emergence of BI in 190 SLE patients who used > 40 mg of prednisolone per day for one month (40). Another similar study reported the development of BI in SLE patients who used 30 mg of prednisolone per day for one month (45). Moreover, positive correlation also exists between BI development in SLE patients and cumulative dose of steroids (10, 50, 53). The incidence of BI was higher in SLE patients who received the cumu-
ative dose of prednisolone at 1 and 4 months compared to the control group (10). In renal transplant cases, the incidence of femoral head BI was also higher when larger total steroid dose was used during first 2 months of therapy (50, 53).

**Initiation**

Table 1 describes the onset time of BI after commencing of steroid treatment. It is evident from the tabulated data that development of BI commences as early as 3.1 months of steroid usage (54-57). It can be concluded that patients and their physicians should remain cautious for the risk of BI development during the treatment course, mainly during first 12 months of commencement of steroid treatment.

**Progression and regression**

Literature study elaborates various studies about the progression of SIBI after its initiation. Spontaneous regression has been observed in SIBI cases of femoral head, hip and knee (56-59). The serial MRI scan revealed spontaneous incomplete regression in 41.1% of patients who suffered from stage I SIBI of knee showing no subsequent symptoms such as bone tissue failure (59). In another study on knee BI, serial MRI scan showed natural improvement of necrotic region in 45% of patients for 12 months after start of steroid followed by no more improvement in the subsequent years (58). Another follow up study revealed spontaneous size regression in 28% renal transplant patients with hip BI. The spontaneous regression depends upon the stage of BI, for example, the probability of regression of early stage BI is higher than that of later stage. In addition, regression of BI depends upon the time duration between treatment initiation of steroid and diagnosis of BI, i.e., the probability of regression of early diagnosed BI is higher than that of later diagnosed after start of steroid therapy. Moreover, the regression of BI is not influenced by the size and site of necrosis (59).

**Pharmaceutical option for treatment**

Pharmaceutical strategy for treatment of SIBI involves the use of various pharmacologically active compounds including bisphosphonates, hyperbaric oxygen (HBO), coenzyme Q10, erythropoietin, anti-hyperlipidemics, anticoagulants, antioxidants and tissue repair protein (66-87).

**Bisphosphonates**

Though bisphosphonates are unable to stop progressiveness of joint destruction; however, these compounds are useful for improving clinical and pain status of patients with steroid-induced bone infarction (SIBI) (60). Pharmacologically, bisphosphonates promote osteoclasts apoptosis and reduces resorption of the osteoclasts. Moreover, the apoptosis of osteoblasts and osteocytes is also inhibited by bisphosphonates. On the other hand, clinical findings and animal studies have revealed that usage of bisphosphonate leads to development of osteonecrosis of the jaw (61, 62). Due to such unwanted effects, further investigations involving larger number of patients should be done for loss-benefit analysis to check therapeutic efficacy of bisphosphonates in SIBI patients (63). Patient’s movement in SI-BN has been made pain-free by using alendronate, which is observed to be capable of inhibiting bone marrow edema (64-66). However, alendronate is not found therapeutically effective in another study conducted in SIBI for 25 weeks. This study showed 7% and 76% therapeutic effectiveness of alendronate and placebo treatment, respectively (67, 68).

**Hyperbaric oxygen**

Patient’s mobility in SIBI has also been improved by using hyperbaric oxygen (69). The mode of analgesic action of hyperbaric oxygen involves the improvement in oxygen supply to the hypoxic tissues resulting in suppression of edema leading to vasoconstriction (70).

Table 1. Time of onset of BI after initiation of high-dose steroid treatment.

<table>
<thead>
<tr>
<th>No.</th>
<th>Total number of patients</th>
<th>Number of patients with BI detected after initiation of treatment</th>
<th>Time of onset of BI after initiation of steroid</th>
<th>BI type</th>
<th>Evidence</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22</td>
<td>21</td>
<td>12 months</td>
<td>Femoral head</td>
<td>MRI</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>32</td>
<td>3.1 months</td>
<td>Hips and knees</td>
<td>MRI</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>48</td>
<td>3.6 months</td>
<td>Femoral head</td>
<td>MRI</td>
<td>57</td>
</tr>
</tbody>
</table>
Coenzyme Q₁₀

Coenzyme Q₁₀ is a lipophilic vitamin-like potent antioxidant. Kömürçü et al. studied the prophylactic effect of coenzyme Q₁₀ in 20 Sprague-Dawley rats with methylprednisolone acetate induced-BI. After 30 days, the hematological examinations revealed the restored normal levels of blood glutathione and malondialdehyde in rats treated with coenzyme Q₁₀ in comparison to control group (not treated with coenzyme Q₁₀). Conclusively, coenzyme Q₁₀ was found to have preventive role in the development of SIBI, possibly due to its antioxidant property (71).

Erythropoietin

Keeping in mind anti-apoptotic and tissue protective role of erythropoietin, Chen et al. tested this compound for managing methylprednisolone acetate induced-BI in rats. Beside reduced expression of caspase-3, the findings of this study reported the improvement in histological state of femoral head in erythropoietin treated rats (test group) as compared to that of control group rats (rats with SIBI but not treated with erythropoietin). Moreover, the preventive role of erythropoietin against SIBI was also observed in test group. The authors also mentioned inhibition of apoptosis of osteocytes and osteoblasts and enhanced expression of vascular endothelial growth factor as the mechanism of anti-ISBI (anti-steroid-induced bone infarction) activity of erythropoietin (72, 73).

Antihyperlipidemics and anticoagulants

The development of SIBI may also be prevented through statin treatment (74, 75). For example, significant reduction in bone fat volume and serum lipid levels, in comparison to the control group, has been reported in rabbits with SIBI after treating with lovastatin (76). According to another study, the usage of cholesterol- and lanolin-rich diets may decrease the incidence of SIBI in comparison to control group of rabbits (77). Since SIBI progression depends on combination of hyperlipidemia and abnormal coagulation, antihyperlipidemics and anticoagulants were simultaneously administered to model rabbits with SIBI to test their therapeutic effectiveness. It resulted in significantly lower incidence of SIBI as compared to that of individual treatments (78, 79). Jiang et al. reported that pravastatin may be used for prevention of SIBI in rats. Mechanistically, pravastatin inhibits PPARγ expression and activate Wnt signaling pathway (80). Thus, antihyperlipidemic and anticoagulant drugs may be suggested to treat SIBI.

Antioxidant treatment

The usage of steroids leads to the induction of oxidative stress, which results in the development of SIBI. It can, therefore, be managed by using the antioxidants (81, 82). In this context, significant reduction in the incidence of SIBI and oxidative stress by using vitamin E in model animals has been observed (83, 84). The possible mode of action
might be the oxidative stress reducing action of Vitamin E on blood and blood vessels, which leads to the anticoagulant effect.

**Iloprost**

Iloprost is a synthetic analogue of prostacyclin PGI2. It acts as a vasodilator and has been proposed to be a suitable therapeutic agent at the early stages of SIBI (85, 86). The mode of action of iloprost involves vascular dilation restoring the blood flow to necrotic region.

**Tissue repair protein**

Ding et al. transplanted HIF (hypoxia inducible factor)-1α transgenic bone marrow cells and noted the improvement in bone regeneration in necrotic regions of SIBI. They attributed tissue repair function of this protein to enhanced expression of mRNA of osteogenic genes and osteogenic differentiation (87).

**CONCLUSION**

Though above stated studies propose some pharmaceutical agents as possible remedy for SIBI, but all of these strategies have serious limitations. Thus, it can be concluded that no efficient pharmaceutical remedy is yet discovered or introduced. The possible fact might be that the exact pathology of SIBI is still undiscovered. Many factors are found to be responsible for SIBI development; therefore, there are multiple biomarkers of this disease. Conclusively, protein-protein interactions should be conducted with the help of computational chemistry and new therapeutic agents should be introduced.

**REFERENCES**


Received: 24. 03. 2015
Hispidulin [5,7-dihydroxy-2-(4-hydroxyphenyl)-6-methoxy-4H-chromen-4-one or 6-methoxy-5,7,4′-trihydroxyflavone or 6-methoxyapigenin or dinatin] is a polyphenolic phytocompound belonging to the flavone class of flavonoids (1). Flavones are generally crafted in herbs and cereals. Recently, flavones have grabbed scientific and public attention because of their reputed beneficial results against certain cancers, atherosclerosis and osteoporosis. Hispidulin, a bioactive flavone, has been reported as an effective anticancer agent and the strongest ligand of benzodiazepine (BZD) site of GABAA receptor (2-4).

Pharmacokinetics of hispidulin
Pharmacokinetics plays an important role in deciding about future drugs. It depends on absorption, distribution, metabolism, excretion and toxicity. For oral absorption of drugs higher polar surface area (PSA) and low molecular weight (m.w.) are considered good. PSA of hispidulin has been reported as 100.12/GC^2, which is appreciably high with m.w. of 300 (20). Regarding distribution of biologically active compound in human body, blood-brain barrier (BBB), permeability and volume of distribution play vital role. An applicable amount of intact bioactive flavonoids, must reach target tissue to produce an *in vivo* effect. Permeability through BBB of chemically synthesized hispidulin in an *in situ* study has been reported comparable to highly penetrating compound - diazepam - with an uptake rate (Kin) of 1.14 mL/min/g (21). Study of absorption and metabolism of flavonoids is essential to assess their impact on human health. Research has been carried out recently on metabolism of bioactive flavonoids (22-24), which generally are absorbed through the intestine and after metabolism non-absorbed material is...
excreted in the bile by colonic microorganisms (25). But recently, in vitro topical permeability of hispidulin has also been tested through pig skin model, in paste or solution form. Hispidulin has been reported for its prominent potential for topical delivery through the skin, with 0.4 mM water solubility and 0.4 nmol cm⁻² h⁻¹ predicted maximum permeation flux (26). Absorption and metabolism of hispidulin plays vital role in its biological properties, therefore, in vivo bioactive forms of hispidulin are important to be discussed. The in vivo properties of hispidulin are clearly known and it is identified as a novel natural ligand for BZD site of central human GABA₃ receptor (27). For metabolic elucidation of hispidulin in large intestine, its biotransformation by the pig cecal microflora has been reported, with almost complete conversion (0.5 mM; t_R = 23.0 min) within 24 h of incubation. Pig cecum model has been reported as suitable ex-vivo replacement of human large intestine (28). Hispidulin degrades into scutellarein through O-demethylation. Scutellarein is an effective α-glucosidase inhibitor (29). Then, 3-(4-hydroxyphenyl)-propionic acid has been reported from scutellarein through ring opening mechanism. Another product (1,2,3,5-tetrahydroxybenzene) was theoretically expected, which transformed into acetyl-CoA and CO₂ via phloroglucinol (21). An investigation (4) has been made for any chemical modification in the structure of hispidulin while uptake by epithelial cells during intestinal absorption. A good permeation of orally administered hispidulin has been reported in its intact form through the Caco-2 cell monolayer. An absence of glucuronidated metabolites confirmed undeteriorated passage of hispidulin through Caco-2 membrane (30).

Excretion of compound from human body depends upon its molecular weight and hydrophilicity. For hispidulin, hydrophilicity has been reported through octanol-water partition coefficient (log P) with a value of 2.479. Compounds with log P value less than 5 are reported to be sufficiently hydrophilic to reach membrane surfaces (20). Toxicological study of hispidulin indicated no tumorigenic or irritation risk, but high reproductive risk is reported. Overall drug likeness of hispidulin has been reported to be 1.11 (20).

**Standard strategies for isolation and pharmacological evaluation of hispidulin**

Hispidulin has attained substantial consideration for its biological and physiological prominence. It has been isolated from different parts of plants, including fresh leaves (31), dry aerial parts (32-35), flowers (7, 36, 37), seeds (38) and roots (39, 40). Literature embraces a huge data reporting isolation of hispidulin and its derivatives from alcoholic extract of several medicinal plants (41-49). Reported data regarding isolation of hispidulin are either based upon random selection of plants (50) or follow-up experimentation (2, 51, 52). A proven critical approach has been adopted towards the isolation of hispidulin, in the compiled literature. Data report the extraction of hispidulin from different
parts of various plants in alcoholic fraction. Established screening lines have been implemented in pharmacological testing of hispidulin (2, 3, 53); models (animals, cell lines etc.) utilized in these tests were very close to final target (patient) (54-56) with parallel evaluation of cytotoxicity (2, 3) through comparison with reference compounds (2, 3, 57). Structure of hispidulin is presented in Figure 1, along with structural elucidation data.

**Total synthesis**

The literature (4) has reported total synthesis of hispidulin (Scheme 2). This synthesis was carried out with 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone 6 and 4-benzyloxybenzoic acid chloride 9 utilizing the Baker Venkataraman reaction (see Scheme 2c). Distinct tracks were employed for the synthesis of both compounds 6 and 9. Synthesis of compound 6 has been reported in five steps, starting from 2,4,6-trihydroxyacetophenone 1 (see Scheme 2a). First step involved conversion of compound 1 into compound 2 through reaction with Me2SO4. Second step involved selective demethylation of 2 to get compound 3 by using AlCl3/chlorobenzene. Third step involved protection of 4-hydroxy group in 3 with a benzyl group to get compound 4. In 4th step, 4 was applied to Elbs persulfate oxidation to get compound 5. Elbs persulfate oxidation secures synthesis of paradiphenols via alkaline potassium persulfate treatment of phenols. The last step gives compound 6 through methylation of hydroxyl group added in the compound during 4th step. Synthesis of compound 9 has been reported in two steps, taking 4-hydroxybenzoic acid 7 as starting material (see Scheme 2b). First step involved protection of hydroxyl group with a benzyl group to obtain compound 8. Second step synthesized compound 9 by chlorinating 8 with oxalyl chloride. Compounds 6 and 9 were reacted yielding benzoyl ester 10, which rearranged itself into compound 11 upon KOH treatment (see Scheme 2c). Compound 11 cyclized to produce compound 12 upon thermal treatment, in the presence of acetic acid/sulfuric acid. Deprotection and selective demethylation of 12 under cold reaction conditions with BCl3/CH2Cl2 resulted in hispidulin 13.

![Figure 1. Hispidulin structure](image)

**Figure 1. Hispidulin structure**

C16H14O6: 302; m.p.: 228-230°C (ref. 115).
UV: 293 (4.23), 331 (3.68); + CH3ONa, 247 (4.07), 330 (4.19); + AlCl3, 225 (4.42), 300 sh (4.12), 316 (4.21), 394 (3.41); + AlCl3/HCl, 225 (4.47), 314 (3.42), 394 (3.41) (116)
IR: 3500 (OH), 1640 (C=O γ-pyrone) (ref. 117)
MS: 302 [M]+, 120 C6H8O
CD: (c 0.001, MeOH) [θ] (nm): -10744 (300) (negative maximum), +13358 (268) (positive maximum) (ref. 115).
1H-NMR: (DMSO-d6, δ, ppm): 2.80 (1H, dd, J = 4.0, H-3 eq), 3.10 (1H, d, J = 12.0, H-3 ax), 3.72 (3H, s, OCH3), 5.42 (1H, dd, J = 12.0 and 4.0, H-2), 6.00 (1H, s, H-8), 6.83 (2H, d, J = 9.0, H-3', 5'), 7.34 (2H, d, J = 9.0, H-2', 6'), 12.20 (1H, s, 5-OH) (ref. 115).
13C NMR (DMSO-d6, δ, ppm): (ref. 56)

<table>
<thead>
<tr>
<th>C-2</th>
<th>78.4</th>
<th>C-8</th>
<th>95.1</th>
<th>C-4'</th>
<th>157.9</th>
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<td>42.1</td>
<td>9</td>
<td>157.6</td>
<td>5'</td>
<td>115.2</td>
</tr>
<tr>
<td>4</td>
<td>196.8</td>
<td>10</td>
<td>102.0</td>
<td>6'</td>
<td>127.9</td>
</tr>
<tr>
<td>5</td>
<td>155.0</td>
<td>1'</td>
<td>129.0</td>
<td>OCH3</td>
<td>59.9</td>
</tr>
<tr>
<td>6</td>
<td>129.0</td>
<td>2'</td>
<td>127.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>159.4</td>
<td>3'</td>
<td>115.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Biological activities of hispidulin

Herbal medication is evolving worldwide, but unsatisfactory certification about their safety or usefulness screens them out. This dilemma, somehow, has been resolved by the improvement and rationalization of appropriate analytical assays. For hispidulin, different pharmacological aspects have been reviewed and summarized in Table 1.

**Anti-oxidant activity and effect on mitochondrial metabolism**

Antioxidant character of flavonoids depends on their ability to hunt free radicals. Biomolecules can easily be spoiled by free radicals through oxidative damage (58, 59). A disproportionation among antioxidants and reactive oxygen species signifies interest of free radicals as ultimate factor, resulting in human body disorders (60, 61). Hispidulin, as an antioxidant, fights against free radicals (oxidizing agents) by making electronic dealings with biomolecules in cells (62). On the basis of fact that structure plays vital role in determining competency of antioxidants (63), two theoretically feasible reaction mechanisms have been calculated and reported from quantum data of hispidulin (1). First mechanism is related to hydrogen removal from hydroxyl groups, and 4-benzylxoxybenzoic acid chloride.

Scheme 2. Total synthesis of hispidulin through Baker Venkataraman reaction using 4-benzyloxy-2,3-dimethoxy-6-hydroxycetophenone and 4-benzylxoxybenzoic acid chloride.
Table 1. Medicinal plants rich in hispidulin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Family</th>
<th>Species / Plant</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hispidulin</td>
<td>Asteraceae</td>
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<td></td>
<td>Aegialophila pumila</td>
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<td>Centaurea pichleri</td>
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<td>Centaurea hierapolitana</td>
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<td>Centaurea inermis and C. virgata</td>
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<td></td>
<td>Oregano (Folium origani cretici)</td>
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</tr>
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<td>Sage (Folium salviae officinalis)</td>
<td>(44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Thyme (Folium thymi)</td>
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</tr>
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<td>Compositae</td>
<td>Artemisia species</td>
<td>(4)</td>
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<tr>
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<td>Artemisia vestita</td>
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<td></td>
<td>Centaurea collina</td>
<td>(132)</td>
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<tr>
<td></td>
<td>Verbenaceae</td>
<td>Lantana montevendensis BRIQ.</td>
<td>(56)</td>
</tr>
<tr>
<td>Hispidulin 7-O-glycoside</td>
<td>Plantaginaceae</td>
<td>Plantago asiatica</td>
<td>(45)</td>
</tr>
<tr>
<td>Hispidulin-7-neohesperidoside</td>
<td>Asteraceae</td>
<td>Cirsium japonicum</td>
<td>(48, 53)</td>
</tr>
<tr>
<td>Hispidulin-7-sulfate</td>
<td></td>
<td>Centaurea bracteata (aerial parts)</td>
<td>(49)</td>
</tr>
<tr>
<td>Hispidulin-7-sulfate</td>
<td></td>
<td>Centaurea bracteata (roots)</td>
<td>(133)</td>
</tr>
</tbody>
</table>
which mainly depends on energy required to break O–H bond i.e., bond dissociation energy (BDE). After comparing all OH groups inside hispidulin molecule, it has been reported that most stable radical for hispidulin is 4′-OH with 84.1 kcal/mol BDE energy, whereas 5-OH and 7-OH show 93.8 kcal and 88.3 kcal BDE energy, respectively (1). Molecules that require less energy to break O–H bond, breed stable free radicals and show strong antioxidant behavior:

\[
\text{ROH} + \text{OH} \rightarrow \text{RO}^\cdot + \text{HOH}
\]

Second mechanism narrates transfer of electron from antioxidant to radical species ensuing indirect H-removal. This mechanism depends upon the energy required to craft ROH\(^+\) radical cation, through ionization, as well as on the reactivity of radical cation. Normally, flavonoids with low IP are considered as strong antioxidants (64). Hispidulin, on energy consideration, has been reported to have low IP value i.e., 6.96 eV in comparison to quercetin with IP value of 7.22 eV (1). Thus, hispidulin shows comparable antioxidant behavior to that of quercetin.

\[
\text{ROH} + \text{OH} \rightarrow [\text{ROH}]^\cdot + \text{OH} \rightarrow \text{RO}^\cdot + \text{HOH}
\]

The literature (65) reports the consequences of hispidulin action on mitochondrial activity, and significance of its structure in mitochondrial respiration inhibition. Effect of this flavone on mitochondrial metabolism has been evaluated through polargraphic experiments consuming 200 μM of each flavone and mitochondrial oxidation medium. An investigation of enzymatic complexes activity in respiratory chain indicated that complex I and III provide effective reaction site (66). Hispidulin activated changes in mitochondrial behavior have been reported at various concentrations, i.e., 100, 150 and 200 μM dilutions reduce membrane electric potential and 75–200 μM hispidulin dose effects in mitochondrial swelling. These results correlate mitochondrial enzymatic inhibition with particular flavone structure (67). The effect of hispidulin on isolated mitochondria has also been studied (68), where 50-200 μM hispidulin has been reported to reduce mitochondrial oxygen consumption for state III, up to 42% and 27% for glutamate and succinate, respectively. Moreover, ADP to oxygen ratio decrease has also been reported (69). Along with that, hispidulin (75-200 μM) has been described to promote rate of oxygen consumption in complex IV using glutamate and succinate as the substrates. State III respiration inhibition was spotted, comparatively prominent, for succinate as substrate. Moreover, hispidulin (200 μM) effects mitochondrial respiration in the presence of glutamate to consume more oxygen in state IV (69). Electronic considerations of hispidulin structure not only style its charge distribution but also define its biological individuality. This structural speciality plays vital role in promoting its antioxidant profile. So, a better exploitation of hispidulin is anticipated in pharmacological and food related fields.

**Anticancer activity**

Hispidulin has the potential to control tumor progression and angiogenesis (3). In this study, mice have been induced with PANC-1 (5 × 10⁶ each mouse) cancer cells from human pancreas, till tumor extended up to 50 mm³. Then, they were divided into two groups (sample mice group and reference mice group) on the basis of daily-injected dosage of 20 mg/kg, with or without hispidulin, respectively. _In vivo_ analysis testified that hispidulin suppresses tumor growth of human pancreas in xenograft mice, without any toxic effect on animal’s weight. _In vitro_ cytotoxic analysis of hispidulin on pancreatic cancer cells and human umbilical vascular endothelial cells (HUVECs) showed receptiveness of HUVECs. This indicated prominent effect of hispidulin on angiogenesis. The IC₅₀ value of hispidulin has been reported to be 20 μM in HUVECs. _Ex vivo_ and _in vivo_ suppression of aortic rings along with corneal neovascular growth stimulated by vascular endothelial growth factor (VEGF) has been reported. Interaction of hispidulin with distinctive molecules in HUVECs were analyzed and a suppression of VEGF-induced activation of VEGF receptor 2, PI3K, Akt, mTOR has been reported. In another study (55), _in vitro_ anticancer effects of hispidulin on human esophageal, nasopharyngeal and colon cells have been reported, using Sarcoma-180 (S180) and Hepatoma-22 (H22) transplantation methods. Broad range dosage of hispidulin (2.5, 5, 10 mg/kg) was implemented for 10 days. MTT essay reported the inhibition of tumor at 30-100 μg/mL of hispidulin in dose dependent manner. The reported tumor inhibition rate was 25.7~67.7% (for S180) and 33.8~75.6% (for H22). The literature (54, 56) also reports _in vitro_ activity of hispidulin against lymphatic, colon, breast, lung, gastric and uterus cancer in human beings. The anti-cancer effect of hispidulin on human gastric cancer cells against commercial medicines like rutin and aspirin was investigated (2). Time and concentration dependent essays testified an appreciably superior anticancer behavior of hispidulin, with IC₅₀ value of 20 μM at 72 h treatment in comparison to rutin with IC₅₀ value of over 500 μM. Aspirin exhibited minute inhibitory effects on gastric cancer cells (IC₅₀ value calcu-
Thorough “proof-of-concept” (76) about the efficacy of hispidulin in antitumor activity has been compiled from in vitro and in vivo studies, with applicable IC_{50} values, in the presence of appropriate controls against commercially available drugs. Therefore, hispidulin is favorable contender on forthcoming anticancer drug development platform.

**Antiepileptic activity**

Epilepsy is a very common brain disease that disturbs about 2% of world population. Available antiepileptic drugs on the market principally work on transmitter receptors and ion channels. Roughly 30% of epileptic patients do not use these drugs, due to undesirable side effects (77). Hence, harmless and efficient antiepileptic drugs of natural product origin were strongly required, particularly to advance innovative epileptic treatments. Various scientists in this scenario had worked on hispidulin (78, 79).

Glutamate is an important neurotransmitter, in mammalian central nervous system (CNS) proficient to accelerate physiological or psychological activity. Excess of glutamate has verified association with epilepsy (80); a sudden surge of electrical activity in the brain of experimental rats has been reported upon treatment with glutamate receptor agonists (81); contrariwise, antiepileptic behavior and drop in seizure-induced brain damage has been reported in experimental animals upon treatment with glutamate receptor antagonists (82). Additionally, human epileptic patients have been reported with enhanced glutamate level (83, 84), signifying excess of glutamate as a cause of epilepsy. The reported mechanism (57) arbitrated by decline in glutamate release through exocytosis (Ca^{2+}-dependent). An investigation was made to check effect of hispidulin on endogenous glutamate release to explore possible mechanisms. It was found that hispidulin constrained glutamate release induced by K^+ channel blocker 4-aminopyridine (4-AP) (85). Hispidulin (10 µM) has been reported to enhance γ-aminobutyric acid (GABA_γ) receptor activity by 65 ± 17% (4). Stimulation of GABA_γ receptor prevented voltage-dependent Ca^{2+} influx and glutamate release from nerve terminals (86). Comparative analysis of glutamate release in the presence of 4-AP (control) alone as well as 4-AP with hispidulin has been reported (57) showing a substantial reduction in glutamate release. Treatment with hispidulin (30 µM) reduced glutamate from 7.3 ± 0.1 nmol/mg to 3.6 ± 0.4 nmol/mg per 5 min; whereas 80% inhibition was observed with 100 µM concentration of hispidulin. Using dose-response relationship, IC_{50} value of hispidulin...
for glutamate release inhibition has been reported as 22 µM. This signifies that a control over glutamate neurotransmission may lead to possible solution for epileptic behavior.

Neural overexcitation or scarce inhibition, generates epileptic attacks due to hyper-synchronous electrical current. Inhibition controlled by GABA<sub>α</sub>-receptors augments in the presence of benzodiazepine (BZD) ligands. Thus, classical BZD ligands like diazepam show effective anticonvulsant activity (87). Flavonoids are reported for their effects on CNS (88). Hispidulin, being a flavonoid, has been reported (27) for antiepileptic activity through binding inhibition of flumazenil with BZD site of GABA<sub>α</sub> receptor with IC<sub>50</sub> value of 1.3 µM. A recent study (89) has reported hispidulin as a potent ligand for BZD site of human GABA<sub>α</sub> receptor, with 81% inhibition of maximal GABA<sub>α</sub> response, showing strongest binding activity to BZD site comparative to ursolic acid, camosol, oleamolic acid, saligenin, rosmanol and cirsimaritin. Reported data about hispidulin neuropharmacology, particularly control over epileptic activity through interaction with GABA<sub>α</sub> receptors, indicate its potential to cope with different neurological and psychiatric disorders.

**Anti-hypnotic activity**

Prolyl oligopeptidase (POP) enzyme contributes in numerous features of CNS function. Noteworthy increase in POP may cause depression, anxiety, anorexia, Parkinson’s disease, schizophrenia and different additional neurological disorders (90). Recently, POP inhibition by different fraction isolated from *S. racemosa* Pers. has been reported (91), with IC<sub>50</sub> values from 18.2 to 30.3 µg/mL. Fractionation resulted in lupeol, oroxylin A, oroxyloside and hispidulin. The inhibitory assays at 100 µM concentration of each compound showed that hispidulin inhibited 43% of total POP activity compared to lupeol (5%), oroxylin A (20%) and oroxyloside (34%). Thus, inhibitory effects of hispidulin suggest the compound as valuable lead for a variety of brain disorders, such as schizophrenia, bipolar affect and Alzheimer’s disease (92).

Sleep disorder destroys not only the cognitive function but also the immune system (93, 94). Insomnia is world spread sleep disorder, effecting chronically 10ñ15% of grown up population (95). Medicinal plants with sedative effect target BZD site of GABA<sub>α</sub> receptor (87), which maintains the equilibrium in neuronal excitation and inhibition (89), to regulate sleep. Peak sedative-hypnotics are targeted through BZD binding spot of GABA<sub>α</sub> receptor (96). Hyperpolarization of membrane, by allowing a Cl<sup>-</sup> influx, induced by GABA<sub>α</sub> is mainly initiated by BZD sites, which hangs up neurotransmission. In this way, BZD sites harvest sedative-hypnotic, anxiolytic and anticonvulsant events (97). The literature (98) has reported sedative-hypnotic activity of hispidulin. Plentiful flavonoids from terrestrial plants have been reported with their affinity for BZD site of GABA<sub>α</sub> receptors (99). In *in vitro* analysis of different substitutions particularly on flavone structure for their affinity to BZD site of GABA<sub>α</sub> receptor has also been reported (100); an increase in binding affinity has been stated for 6-methoxylation (hispidulin), whereas 7- or 3-methoxylation resulted in significant decrease in activity (see Fig. 2). This makes hispidulin superior compared to crisimaritin (7-methoxy compound) and galingalin-3-methyl ether (3-methoxy compound) in binding affinity for BZD site of GABA<sub>α</sub> receptor. Hispidulin has also been reported from sedative plants with binding affinity value of 8 µM (89).

![Figure 2. Effect of methoxy substitution on BZD binding affinity](image-url)
Anti-osteoclastogenesis activity

Human skeleton strength depends on equilibrium between bone resorption (osteoclasts) and bone formation (osteoblasts). In osteoporosis, equilibrium shifts towards osteoclasts, and bone resorption surpasses bone formation (101), particularly in females with estrogen deficiency (102). Mechanistic pathway study revealed that osteoclast inhibition was triggered primarily by disturbance in nuclear factor κB (NF-κB), Jun N-terminal kinase (JNK) and mitogen-activated protein kinases (p38) signalling rather than extracellular signal-regulated kinases (ERK). Hispidulin tempers osteoclastogenesis and bone resorption (103). Dose dependent osteoclast inhibition has been reported with hispidulin in two different cell cultures. Hispidulin cytotoxicity was done by cell viability essay up to 10 μM without noticeable cell loss, indicating that osteoclast inhibition is not due to its cytotoxic behavior. A meaningful drop in osteoclast specific gene expression, analyzed through Reverse Transcription Polymerase Chain Reaction (RT-PCR) has also been reported for hispidulin in a concentration dependent fashion. Hispidulin is not only an effective inhibitor of bone resorption but also a remedy to control abnormal bone lysis (104, 105).

Modern development of genetic and biological mechanistic approach in bone resorption has broadened the therapeutic concepts for antiresorptive usages. Available commercial medications may cause severe side effects like breast cancer, endometritis, thromboembolism, hypercalcemia or osteonecrosis (106, 107). Therefore, new drugs must be assessed in certain safety aspects; they should not be accumulated within bone, and have not extended existence in plasma so as to counteract the anabolic endeavor.

Anti-inflammatory activity

Delayed type hypersensitivity (DTH) is immune response triggering inflammatory diseases, by producing many proinflammatory cytokines. These disorders are normally treated with immunosuppressants, which have severe side effects including cytotoxicity. Hispidulin has proven antiinflammatory effects as was mentioned in various articles (3, 67, 102, 108-110).

Hepatoprotective activity

The diverse functionality of hispidulin extends to hepatoprotective effects as well, which have been reported on CCl4 intoxicated mice (111). These effects were evaluated through concentration control of two serum enzymes, named AST (aspartate transaminase) and ALT (alanine transaminase), which highlight hepatic injury in high concentration.

At a dose of 300 mg/kg of hispidulin, a decrease in AST from 70 U/L to 36 U/L and in ALT from 244 U/L to 35 U/L has been reported (111). Another study (112) has reported an association between liver injury and hepatic lipid peroxidation. Ferrandiz et al. (113) have reported positive effects of hispidulin on inhibition of hepatotoxicity induced by bromobenzene. It has been stated that bromobenzene did not affect liver weight but causes necrosis, which has been gauged by serum alanine aminotransferase (SALT) level, lipid peroxidation as malondialdehyde (MDA) equivalents and protein contents through reduced glutathione (GSH). Varied dose range (50-150 mg/kg) has been implemented to validate hepatoprotective activity of hispidulin. Reported results have shown nine times increase in SALT activity, four times increase in lipid peroxidation and five times decrease in GSH level, in intoxicated animals relative to control (non-intoxicat-ed). At a dose of 150 mg/kg of hispidulin, reported data state a decrease in SALT level from 441 to 213 (U/L), a decrease in lipid peroxidation from 271 to 104 (pmol MDA/mg protein) and a decrease in GSH from 8.4 to 5.6 (nmol/mg protein). Comparatively, non-intoxicated controls showed SALT level at 65 (U/L), lipid peroxidation at 68 (pmol MDA/mg protein) and GSH level at 42 (nmol/mg protein). Hispidulin upon comparison with reference compound - N-acetyl-L-cysteine, at the same dose, has presented promising inhibition of liver injury as well as lipid peroxidation. The promising outcomes for in vivo hepatoprotective pursuit of hispidulin advocates its controlled clinical studies, and indicates its candidature as future drug.

CONCLUSION

Since new drug development from natural products requires quality standards not only at isolation stage but also in pharmacological screening; the data has been compiled with standard evaluation procedures, in comparison with positive and negative controls. This review article evidences imperative therapeutic effects of hispidulin for distinctive biological activities, suggesting its potential utilization in medicine, not only on cultural, anthropological and ethnobiological basis but also on pharmacological studies. After glimpsing the reported data, a logical query arises about pharmacological future of hispidulin as a potential medicine, keeping its cytotoxic consequences in mind.

Percent inhibition values (Table 2) clearly indicate that anticancer and antiepileptic activities of
Table 2: Pharmacological aspects of hispidulin.

<table>
<thead>
<tr>
<th>No.</th>
<th>Activity</th>
<th>IC₅₀(µM)</th>
<th>% Inhibition (at conc.)</th>
<th>Methodology</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>Antioxidant</td>
<td>NT</td>
<td>50 (10⁻⁵ M)</td>
<td>Lipid peroxidation inhibition</td>
<td>(134)</td>
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<td>2</td>
<td>Anticancer</td>
<td>20</td>
<td>80* (100 µM)</td>
<td>MTS assay</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 (at 72 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90* (100 µM)</td>
<td>MTT assay</td>
<td>(2)</td>
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<td>3</td>
<td>Antiepileptic</td>
<td>1.3</td>
<td>NT</td>
<td>H-flumazenil-BZD binding inhibition</td>
<td>(27)</td>
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<td></td>
<td></td>
<td>8</td>
<td></td>
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<td></td>
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<td>81 (100 µM)</td>
<td>Maximal GABA response</td>
<td>(89, 135)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>22</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td>80 (100 µM)</td>
<td>Glutamate release inhibition</td>
<td>(57)</td>
</tr>
<tr>
<td>4</td>
<td>Anti-hypnotic</td>
<td>NT</td>
<td>2 (10 nM)</td>
<td>POP inhibition</td>
<td>(91)</td>
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<td></td>
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<td>3 (1 µM)</td>
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<td></td>
<td>43 (100 µM)</td>
<td></td>
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<tr>
<td>5</td>
<td>Cytotoxic</td>
<td>15.5°(Lung Adenocarcinoma)</td>
<td>NT</td>
<td>MTT colorimetric assay</td>
<td>(71)</td>
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<td></td>
<td>7.6°(Breast Adenocarcinoma)</td>
<td>NT</td>
<td>MTT colorimetric assay</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10.4°(Cervical Adenocarcinoma)</td>
<td>NT</td>
<td>MTT colorimetric assay</td>
<td>(71)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 (Cervical Adenocarcinoma)</td>
<td>NT</td>
<td>MTT colorimetric assay</td>
<td>(50)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NT</td>
<td>43 (200 µM)</td>
<td>NADH oxidase inhibition</td>
<td>(65)</td>
</tr>
<tr>
<td>6</td>
<td>Anti-influenza</td>
<td>13.9°</td>
<td>NT</td>
<td>Influenza H₃N₂ virus neuraminidase inhibition</td>
<td>(52)</td>
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<tr>
<td>7</td>
<td>Antidiabetic</td>
<td>0.49</td>
<td>NT</td>
<td>DPP-IV inhibition</td>
<td>(136)</td>
</tr>
</tbody>
</table>

* Digital values estimated from graphical data presented in relevant articles. °µg/mL; NT = not tested; POP = prolyl oligopeptidase.
Pharmacological assessment of hispidulin - a natural bioactive flavone

Hispidulin are fairly higher than its cytotoxic effect even at half dosage. Moreover, it can be comprehended that cytotoxicity of hispidulin becomes insignificant at the concentrations reported for anticancer and antiepileptic activities. Hispidulin has less cytotoxic behavior and better anticancer effect than available commercial medicines. It has a proven efficacy in comparison to commercial COX-2 inhibitors. Moreover, noticeable reduction in volume and weight of cancer polyp, without affecting animal weight, signposts complimentary effect of hispidulin on normal body cells. Cytotoxic effect of hispidulin has been tested against the reference anticancer drug - adriamycin, and reported values for hispidulin are less than those of the reference drug (71).

Flavones, out of entire flavonoid group, are known to be the best for their effects on CNS, as flavones have highest binding affinity for BZD site of GABA_A receptors, and out of different flavones, hispidulin has shown maximum binding affinity (100).

DFT study has shown antioxidant capacity of hispidulin comparable to quercetin, indicating that radical scavenging nature of hispidulin has convincing competency to fight against reactive oxidizing species.

Structural characterization of hispidulin, particularly methoxy group at position-6 and hydroxyl group at position-7, upon comparative analyses against other members of the class show promising anticancer, antihypnotic and antiepileptic behavior. Methoxy group at C-6 (in hispidulin) compared to hydrogen at the same position has 760 folds higher pharmacological output. Similarly, hydroxyl group at C-7 (in hispidulin) is four times better than the hydrogen and 350 times better than methoxy group, for BZD binding affinity.

The most significant argument is that severe side effects have been reported for commercially available drugs of epilepsy and osteoclastogenesis, but hispidulin treatment reports no such side effect. Commercial antosteoclastogenesis drugs are reported to cause cancer (106, 107), while hispidulin behaves as an anticancer agent. Similarly, antiepileptic drugs show side effects like memory impairment (77), but hispidulin treatment results otherwise (114). These evidences underline the importance of research endurance on distinctive therapeutic activities of hispidulin. Petite clinical trials but convincing in vitro, in vivo and ex vivo literature on pharmacological aspects of hispidulin ensures that scientific platform is infantile but influential to provide technical foundation for clinical trials in any direction. It is worth mentioning that hispidulin does not show any violation to Lipinski’s rule of five, as is evident from the literature reported in pharmacokinetic studies.

REFERENCES

Pharmacological assessment of hispidulin - a natural bioactive flavone


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Among many compounds that affect change in concentration of TG (triglycerides) in plasma are selected clofibric acid derivatives. These compounds modify concentration of structural and enzymatic proteins both in plasma, cytoplasm, and other organelles, such as mitochondria or peroxisomes. Elevated concentrations of TG in plasma entails serious health consequences as a development of atherosclerosis, and therefore coronary heart disease and ischemic heart disease. In contrast, clofibric acid derivatives are among very few therapeutic agents lowering TG levels by affecting structural and regulatory proteins of lipoprotein fraction as well as enzymatic and receptor proteins.

TG are esters of glycerol and higher fatty acids. In the body, TG are formed in liver, intestine and in adipose tissue. Then, triglycerides are bound in lipoprotein fractions, which move to plasma. In the body, as a TG are transported most of the long chain fatty acids taken with food. TG constitute lipid material in white adipose tissue (90% of lipid content) as well as reserve and energy materials.

In humans, there are lipoprotein fractions such as chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high-density lipoprotein (HDL). TG-rich are VLDL and chylomicrons. Inactivation of TG by lipoprotein lipase occurs with simultaneous degradation of these fractions and is prerequisite for proper lipid metabolism because it provides fatty acids to all tissues and organs. This process depends on activities of many proteins including apolipoproteins (apo). In conditions when amount of TG fractions in plasma is increased, are used lipid-lowering drugs.

**Fibrates as clofibric acid derivatives**

Clofibric acid derivatives called fibrates, are quite commonly used lipid-lowering drugs, so it is necessary to know beneficial and adverse effects of these compounds on the body. The European Medicines Agency’s Committee for Medicinal Products for Human Use (CHMP) has concluded that benefits of four fibrates such as: bezafibrate, ciprofibrate, fenofibrate and gemfibrozil continue outweigh their risk in treatment of people with blood lipid disorders. According to recommendations of the CHMP fibrates should not be used as first-line drugs, except in patients with severe hypertriglyceridemia and patients who cannot use statins. In this paper, we focused on effect of clofibric acid derivatives on lipid metabolism, in particular on apoproteins and regulatory enzymes.

**Keywords:** clofibric acid derivatives, apo proteins, regulatory enzymes, lipid metabolism

**Abbreviations:**

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necessary to know adverse effects of these compounds on the body. The European Medicines Agency’s Committee for Medicinal Products for Human Use (CHMP) has concluded that benefits of four fibrates such as: bezafibrate, ciprofibrate, fenofibrate and gemfibrozil continue outweigh their risk in treatment of people with blood lipid disorders. According to recommendations of the CHMP, fibrates should not be used as first-line drugs, except in patients with severe hypertriglyceridemia and patients who cannot use statins.

Clofibric acid derivatives impact on hepatocytes in different organelles, especially in nucleus, mitochondrions and peroxisomes (Fig. 1). Most studies relating to the effect of clofibric acid derivatives were performed in order to determine their impact on the activity of peroxisomal enzymes (1-3). For example, in studies conducted on rats it was

![Clofibric acid derivatives and their structural formulas](image-url)
demonstrated that clofibrate acid derivatives cause significant hypertrophy of peroxisome in liver tissue (4, 5). In turn, in humans, it is assumed that peroxisome hypertrophy under the influence of clofibrate occurs only to a limited extent (6). Clofibrate in patients increases amount of liver mitochondrions (7). In consequence, it leads to increase of acetyl-CoA which is involved in Krebs cycle.

**Structure and mechanism of effects of clofibr acid derivatives**

Clofibrate (KF) was the first one to be used in the early sixties (1967, in the U.S.) but then, due to numerous side effects, it was withdrawn from use (8). Successively, fenofibrate (FF), bezafibrate (BF), ciprofibrate (CF) and gemfibrozil (GF) were put on the market in the seventies. Fibrates are halogen derivatives and contain chlorine atom (excluding gemfibrozil). Their chemical structure refers to short chain fatty acids. In molecules of KF, FF and BF one of substituents is chlorine connected by covalent bond to aromatic ring. It affects biological function of clofibr acid derivatives. Replacement of chlorine atom in the molecule of clofibrate acid with extensive substituent such as 4-chlorobenzoyl in case of FF, or 2-(4-chlorobenzamide)-ethyl in case of BF increased lipophilicity of these compounds (9, 10).

Micronized FF and CF, currently used in clinical practice, cause less severe side effects. Micronization - size of particle less than 50 µm, results in improved absorption compared to a standard substance. It increases about 30% bioavailability of drug. Fibrates are very well absorbed into blood from gastrointestinal tract. In blood, about 95% of fibrates are bound to plasma albumin. BF, CF and GF reach maximum concentration after 2 h while FF after 4-6 h. The metabolism of these compounds takes place in the liver whereas they are excreted in 60-90% by kidney. Biological half-life of FF is equal to 22 h; BF - 2 h; CF - 18 h and GF - 1.1 h (Table 1).

Table 1. Pharmacokinetics and metabolism of selected clofibr acid derivatives.

<table>
<thead>
<tr>
<th>Fibrates</th>
<th>Degree of protein binding [%]</th>
<th>Half-life [h]</th>
<th>Excretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenofibrate</td>
<td>&gt; 99</td>
<td>19–27*</td>
<td>100% renal as glucuronides form</td>
</tr>
<tr>
<td>Clofibrate</td>
<td>96</td>
<td>18–22**</td>
<td>100% renal as metabolized form</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>95</td>
<td>1.5–3.0***</td>
<td>100% renal as unchanged form</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>98</td>
<td>1.1–3.7****</td>
<td>100% renal as metabolized and glucuronides form</td>
</tr>
<tr>
<td>Ciprofibrate</td>
<td>99</td>
<td>70–81*****</td>
<td>100% renal as glucuronides form</td>
</tr>
</tbody>
</table>

* The values are based on the publications: *(11, 13), **(12, 13), ***(13), ****(13, 58, 59), ******(60).
Knowledge of mechanisms of action of fibrates has been extended and thus clearly explained at the time when, in 1990, was discovered nuclear receptor activated by peroxisome proliferator, so-called, PPAR (peroxisome proliferator activated receptor) (13). It includes three types of PPAR: α, δ (also called β, NUC-1 or FAAR) and γ, each of which are encoded by separate genes (16). These receptors are present in various tissues, and are transcription factors that allow signal transmitted from fat-soluble substances (natural fatty acids, hormones such as glucocorticoids, vitamins, some lipid-lowering drugs) to target sites, which are genes responsible for synthesis of proteins involved in lipid metabolism (15, 17).

Fibrates, after entering to cytoplasm, connect to specific transporter proteins, and then are passed to nucleus where bind to PPAR. Similarly to Wy 14.643 (4-chloro-6-[(2,3-dimethylphenyl)amino]-2-pyrimidinyl][thio]-acetic acid (pirinix acid), unsaturated fatty acids and their fibrate derivatives are ligands of PPARα receptor. After activation, PPAR-α forms a heterodimer with a nuclear receptor for 9-cis retinoic acid: RXR. Then, thus formed transcription factor PPAR/RXR combines with a strictly defined sequence of DNA known as PPRE (PPAR response-element).

Effects of fibrates on apoproteins and transporting and regulator enzymes

These effects cause a change in regulation of gene expression for particular proteins involved in lipid metabolism such as LPL, HL (hepatic lipase), protein apoA-I, A-II, AV, apoB-100, apoC-III, and apoC-IV.
Coronary effect of fibrates on proteins and enzymes which hydrolyze triacylglycerols

Fibrates activate degradation of TG-rich fractions, such as VLDL and chylomicrons by increasing their lipolysis. In addition, this activates LPL or causes an increase in availability of these fractions to lipolysis catalyzed by this enzyme. Lowering concentration of apoC-III, which is an inhibitor of LPL, allows for efficient action of the enzyme (19). FF results in inhibition of transport of cholesterol esters HDL<sub>3</sub> to VLDL fraction, leading to an increase in HDL-C (22). FF causes a slight decrease in apoE mRNA expression, but this effect was not observed for KF or GF (23). It has been shown that fibrates increase concentration of apoB-100 which is structural protein of VLDL (24). Administration of KF as well as FF to animals caused an increase of hepatic apoB mRNA (apoB-100), but there was no such influence on intestinal apoB mRNA (apoB-48) (24). Clofibric acid increases elimination of LDL particles, since it affects on forming complexes LDL with LDLR and LRP receptor (LDL receptor-related protein) (25, 26). Simultaneously, under influence of fibrates, takes place intensification of synthesis and activity of these receptors in the membranes of hepatocytes. In plasma, in fraction of HDL, it is a protein PLTP which is transporting phospholipids between fraction of VLDL and chylomicrons as well as subfraction of pre-β-HDL. Fibrates accelerate synthesis of protein mainly in liver (27).

In liver, clofibrac acid derivatives increase rate of fatty acids uptake and their conversion into acyl-CoA because they activate fatty acids transporter protein (FATP) and acyl-CoA synthetase (28). It leads to formation of large amounts of TG. In such conditions may occur more of VLDL particles. Fibrates induce pyruvate dehydrogenase kinase (PDK) mRNA mainly in skeletal muscle and liver (29). This results in inactivation of PDH complex by receptor proteins such as SR-B1 (scavenger receptor class B) (Tables 2 and 3) (11, 18-21).

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<table>
<thead>
<tr>
<th>Apolipoprotein</th>
<th>Fractions in which occur apoprotein</th>
<th>Functions</th>
<th>Concentration in plasma (mg/dL)</th>
<th>Half-life</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-I</td>
<td>HDL, chylomicrons</td>
<td>the main structural protein of HDL, activator of LCAT</td>
<td>100-150</td>
<td>about 4 days</td>
</tr>
<tr>
<td>ApoA-II</td>
<td>HDL, chylomicrons</td>
<td>structural protein of HDL, inhibitor of HL</td>
<td>30-40</td>
<td>about 4 days</td>
</tr>
<tr>
<td>ApoA-IV</td>
<td>chylomicrons, HDL</td>
<td>facilitates release of chylomicrons from intestine, activator of LCAT</td>
<td>15</td>
<td>about 1 day</td>
</tr>
<tr>
<td>ApoC-I</td>
<td>chylomicrons, VLDL, HDL, IDL</td>
<td>activator of LCAT, inhibits download residual fraction by hepatocytes</td>
<td>6</td>
<td>about 6 h</td>
</tr>
<tr>
<td>ApoC-II</td>
<td>chylomicrons, VLDL, HDL, IDL</td>
<td>activator of LPL</td>
<td>4</td>
<td>about 6 h</td>
</tr>
<tr>
<td>ApoC-III</td>
<td>chylomicrons, VLDL, HDL, IDL</td>
<td>inhibitor of LPL</td>
<td>12</td>
<td>about 6 h</td>
</tr>
<tr>
<td>ApoB-48</td>
<td>chylomicrons, residual chylomicrons</td>
<td>structural protein of chylomicrons</td>
<td>fasting</td>
<td>under 1 h</td>
</tr>
<tr>
<td>ApoB-100</td>
<td>VLDL, IDL, LDL</td>
<td>structural protein of VLDL, IDL, ligand of „B-100,E” receptor</td>
<td>80-100</td>
<td>about 3 days</td>
</tr>
<tr>
<td>ApoD</td>
<td>HDL</td>
<td>transports of free cholesterol</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>ApoE</td>
<td>chylomicrons, residual chylomicrons, VLDL, IDL, HDL</td>
<td>binding of residual fractions of LDLR and LRP</td>
<td>3-7 under 1 day</td>
<td>1 day</td>
</tr>
</tbody>
</table>

Table 3. Summary of apoproteins, their location, functions, concentration and half-life.
phosphorylating enzyme catalyzing irreversible decarboxylation of pyruvate. Limitation of activity of this complex results in inhibition of conversion reaction of pyruvate to acetyl-CoA (29, 30). Under these conditions, β-oxidation pathway is activated, which can be directly induced by fibrates, because they affect expression of enzymatic proteins such as acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase (19, 23, 25, 26). Fibrates, by increasing expression of proteins involved in β-oxidation, limit synthesis of fatty acids.

In the human liver, fibrates stimulate expression of genes encoding structural apolipoproteins of HDL: apoA-I and apoA-II. Thereby, they increase production of pre-β-HDL, resulting in efficient return transport of cholesterol from peripheral tissues. Opposite effects of fibrates on apoA-I and apoA-II were observed in mice and rats (31). In animals, there was a significant decrease in expression of hepatic apoA-I mRNA but not in intestinal tract. Decreased amount of structural protein causes lowering of HDL. In animals, probably reduced synthesis of apoA-I is due to lack of PPRE action (31-33). Similar observations were made by Duez et al. (34). They investigated effect of fibrates on size of atherosclerotic lesion and concentration of apolipoprotein apoA-I and lipid fractions in apoE knockout mice (control group) and in apoE knockout mice with transgenic human apoA-I gene (study group). It was found that in study group fibrates increase level of HDL in plasma, thus received amount of cholesterol from peripheral tissues in humans is higher, whereas in animals is lower. Also expression of apoA-I mRNA - main protein component of HDL in plasma, increased (33).

Morishima et al. (35) as Vu-Dac (31) and others (36, 37) found that transcriptional regulation of apoA-I by PPAR-α is species specific. Administration of fenofibrate in animals resulted in a decrease in expression of hepatic apoA-I mRNA. In humans the effect was opposite.

Fibrates, by activation of PPAR-α, increase expression of SR-B1 receptors mRNA causing intensification of their numbers. SR-B1-mediated in selective uptake CE by hepatocytes from HDL2, and therefore have opposite effect on transport of cholesterol (37, 38). Results of Fu et al. (39) suggest that apoE knockout mice fibrates (CF 0.05%) caused a decrease in expression of hepatic SR-B1 protein, which contributed to overaccumulation of residual lipoprotein in plasma. Similar conclusions have provided Mardones et al. (38), wherein they found no effect of fibrates expression of SR-B1 mRNA in animals. Observations of ciprofibrate (0.2%) and other PPAR-α agonists effect on SR-B1 expression in humans have shown that these drugs, in opposite way than in animals, regulate expression of this protein (38).

Vu-Dac et al. (31) study demonstrated that fibrates effectively decrease level of TG contained in lipoprotein fractions both in humans and rodents. This study has also shown that use of FF for 7 days in transgenic mice with human apoA-I gene increased concentration in plasma of apoA-I to 750% and HDL to 200% (31). Similar results were obtained by Berthou et al. (40). In addition, it was noted importance of dose and duration of therapy. Maximum effects of FF were obtained at a dose of 0.5% applied for seven days (40). Steals et al. (25) and Elisaf (41) concluded that fibrates also decrease concentration of cholesterol and TG in plasma by accelerating catabolism of VLDL. In their studies were used transgenic mice with overexpression of human apoA-I gene, thereby showing changes in vivo.

Blanco et al. (42) also conducted research on apoA-I and apoA-II protein. By isolating genes of these proteins they identified their role in lipid metabolism and development of atherosclerosis. They found that apoA-II is a genetic determinant of HDL concentrations. Fibrates through complex PPAR/RXR may affect on expression of apoA-I and apoA-II genes. Peters et al. (19) explained mechanism of PPAR-α receptor activity. In their experiment were used two groups of mice: control and research with isolated PPAR-α gene. Group of mice with isolated PPAR-α receptor (PPARα-/-) showed increased levels of total cholesterol and HDL (approximately by 63%) compared to control (PPAR-α +/-). In turn, composition of HDL particles remained unchanged. Furthermore, in PPAR-α-/- group was observed a slight increase in amount of apoA-I, apoA-II in plasma and its mRNA and thus increased concentration of HDL. Thus, it confirms active role of PPAR-α in control of HDL metabolism. This experiment shown also that in mice PPAR-α +/- both Wv 14.643 and fibrates decrease level of hepatic apoC-III mRNA and TG. Activation of PPAR-α stimulates uptake of fatty acids in liver and conversion to acyl-CoA derivatives. Indeed there is induction of genes such as: fatty acid transport proteins and acyl-CoA synthetase - catalyzing conversion of fatty acids in acyl-CoA (19, 23, 25, 28).

Regulation by fibrates also relates apolipoprotein apoA-V gene, specified by Vu-Dac et al. (43) as a key determinant of level of TG. The experiment
performed on animals (transgenic mice with apoA-V) has demonstrated that fibrates significantly induce apoA-V mRNA. Researchers determined the effect of apoA-V on level of VLDL TG and chylomicrons. ApoA-V decreases secretion of VLDL and chylomicrons but this has not been confirmed by Merkel et al. (44). ApoA-V influences an increase of catabolism of these particles fractions and facilitates their binding to proteoglycan which is joining LPL to cell membrane. Thus, this protein is an activator of LPL. It was also observed that animals with overexpression of apoA-V showed a decreased level of apoC-III. A decrease in apolipoprotein apoC-III inhibiting LPL also reduced concentration of VLDL TG and chylomicrons. Similar conclusions are provided by Oliva et al. (45).

Fu et al. (24) conducted an experiment on LDL receptor knockout mice checking effect of administration of PPAR-α agonist - CF on apoB mRNA expression and lipid parameters. Administration of CF to control group with current LDLR (low-density lipoprotein receptor – LDLR+) resulted in decreased levels of TG and a slightly decreased concentration of free cholesterol (FC) compared to groups that were not receiving CF. In group with LDL receptor knockout (LDLR-) there were observed elevated levels of both TG and FC.

compared to control group (LDLR+). Administration of CF resulted in decreased level of TG in both groups. Increasing concentration of apoB-100 in LDLR knock-out mice explained intensified release into plasma apolipoprotein and retarded clearance of IDL and LDL leading to increased its concentration in plasma. In turn, in LDLR+ group together with an increase in secretion of VLDL containing apoB-100, did not occur accumulation of IDL and LDL. On surface of liver cells were efficient uptake IDL and LDL by LDL receptors (24).

Observation of impact of HF and Wy 14.643 on secretion and circulation of apoB-100 and apoB-48 was also made by Linden et al. (46). They conducted experiment on human cell cultures and isolated rat hepatocytes. In control cells group, predominantly amount ofapoB-100 occurred within VLDL and smaller amount in particles of more dense fraction (i.e., IDL, LDL). Administration of clofibrate or Wy 14.643 resulted both in increase in relative amount of apoB-100 in VLDL and proportion of apoB-100 within IDL and LDL fractions. In contrast, there was not such effect on apoB-48. They also found decreased biosynthesis of TG. These effects occurred after administration to cell both KF and Wy 14.643.

Opposite conclusions about effects of FF on level of apoB-100 delivered Winger et al. (47). They studied effect of FF on lipid parameters in obese rhesus monkeys (Macaca mulatta). The animals had increased level of TG and decreased level of HDL-cholesterol. After administration of this drug, it was observed a decrease levels of TG by 55%, LDL cholesterol by 27% while HDL-cholesterol increased by 35%. It was also pointed a trend of decreasing concentration of apoB-100 and apoC-III during FF therapy in these animals. The effect of decrease concentration of TG and increase concentration of HDL-cholesterol was explained by the fact that fibrates intensify lipoprotein lipolysis. Gemfibrozil does not affect as efficiently on lowering concentration of apoC-III as FF and BF. However, GF does not decrease concentration of apoE (48, 49).

In studies of Coste and Rodriguez (50), it was found that both concentration in plasma and ratio of synthesis of apoC-III are positively correlated with level of TG in plasma in normo- as well as in hyper-triglyceridemia. Deficiency of apoC-III is observed in humans with intensified catabolism of VLDL whereas an excess of this apolipoprotein occured with hyper-triglyceridemia. It was confirmed that regulation of apoC-III gene expression through use of fibrates takes place in transcription (25, 37, 48, 49, 51).

Raspe et al. (52) demonstrated that fibrates induce expression of Rev-erb α receptor that specifically decreases activity of apoC-III promoter. In this experiment, were used mice both with lack and overexpression of Rev-erb α receptor (Rev-erb α-/-; Rev-erb α +/-). The presence of this receptor decreased activity of apoC-III promoter, while in Rev-erb α-/- group was shown an increase in hepatic apoC-III mRNA as well as in concentration of this apolipoprotein in plasma and elevated level of TG contained in VLDL fraction. The metabolism of some lipoprotein fractions in plasma with an indication of effect of fibrates are shown in Figure 2.

Degradation of TG with participation of these proteins is a several-step process, which involves a number of conditions to allow proper lipid metabolism in the system. TG contained in chylomicrons allow transport of fatty acids (including exogenous such as: linoleic, linolenic and arachidonic acid) from intestine to extrahepatic tissues. Fatty acids (including exogenous acids), vitamins A, D, E and K as well as choline and inositol (obtained from food) enter liver along with residual chylomicrons. Vitamins can be stored in hepatocytes. In addition, residual chylomicrons participate in direct and indirect transport of cholesterol to liver in postprandial period (53).

Production of VLDL allows to remove insoluble TG and CE (cholesterol esters) off liver. Between digestive periods, VLDL-TG are beside adipose tissue a source of fatty acids for extrahepatic tissues. In liver, part of cholesterol taken from particles of LDL and other residual fraction undergoes biotransformation to bile acids. This allows excretion of cholesterol with bile from the system. In addition, cholesterol that gets into hepatocytes modifies rate of VLDL formation. HDL as the richest in protein fraction, provides mainly apolipoproteins, which are necessary for degradation of chylomicrons and VLDL in plasma. Moreover, within HDL particles occurs esterification of free cholesterol, which is moved from other fractions of plasma lipoprotein or is taken from tissues with participation of ABCA1 protein (54, 55, 57).

HDL fraction, especially HDL2, and HDL3, are involved in direct transport of cholesterol and phospholipids from extrahepatic tissues to liver. Intensification of synthesis of all apoA proteins leads to easier formation of native HDL particles in liver, and consequently, to increase concentration of this fraction in plasma. The effect of fibrates and protein apoA-V is visible in the impact of increasing LPL activity and rate of chylomicrons and VLDL degradation. ApoC-V like fibrates decreases concentration of apoC-III in plasma (45).

Effect of fibrates on lipid profile in plasma reflects decreased levels of TG and LDL as well as
increased concentration of HDL. Lowering concentration of TG is done by regulation of genes expression of proteins involved in its metabolism. To decrease of TG in plasma by fibrates also contributes stimulation of LPL gene expression (which catalyzes hydrolysis of TG) as well as lowering expression of apoC-III gene (19, 45).

In hepatocytes, fibrates intensify expression of enzymes β-oxidation process of fatty acids genes, which results in a decrease in concentration of fatty acids necessary for synthesis of VLDL-TG. Fibrates not only lower but also normalize content of VLDL-TG. Under these conditions, after degradation of VLDL, LDL particles are formed. These drugs have an impact on efficient removal of LDL from plasma, because they facilitate formation of complexes: LDL-LRP and LDL-LDLR (21).

Fibrates also regulate amount of HDL because, these drugs intensify expression of apoA-I and apoA-II genes, which are structural proteins of this fraction. Consequently, there is an increase in concentrations of HDL in plasma and efficient receiving FC from extrahepatic tissues. Effect of these drugs was also noted in regulation of apoB-100 mRNA, because fibrates increased its amount (46).

Effect of fibrates includes not only enzymatic, regulatory and structural proteins but also receptor proteins. These compounds stimulate expression of SR-B1 and ABCA1 receptors genes. The first of them - (SR-B1) is involved in transport of cholesterol from extrahepatic tissues to hepatocytes, while ABCA1 protein mediated in displacement of cholesterol and phospholipids from cells of peripheral tissues and their incorporation into HDL particles (53, 55).

CONCLUSIONS AND PERSPECTIVE

In conclusion, fibrates have a beneficial effect on lipid profile in plasma because of observed decreased concentration of TG both in LDL and VLDL fractions as well as an increase in concentration of HDL. However, there is growing number of publications reporting on incidents of side effects of these drugs. Research groups are charged that studies are conducted too rarely and include only a small number of patients (also animals) which is disproportionate with percentage of patients with coronary-vascular disease. Thus, results may be inadequate in relation to the entire population.

Conflict of interests

All authors declare that they have no conflict of interests.

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REFERENCES

For thousands of years people have used mushrooms for both medicinal and culinary purposes. However, not every edible mushroom is worth eating. Some should be ignored for various reasons. They may have neither taste nor fine consistency, or may have poisonous relatives which are similar in appearance. On the other hand, numerous species of mushrooms are considered delicacies and so are generally appreciated for their taste and aroma. The most frequently harvested mushroom in Polish forests is Yellow chanterelle (chanterelle) – *Cantharellus cibarius* Fr., from the Cantharellaceae family. Chanterelle is an ectomycorrhizal mushroom occurring in Poland. Chanterelle lives in symbiosis with pine, spruce, oak and hornbeam. In cookery, chanterelle is appreciated because of the aroma, taste, firmness and crunchiness of its fruiting bodies. Wild edible mushrooms are widely consumed in Asia, Western Europe and Central America. Chanterelle contains a great number of carbohydrates and proteins and a low amount of fat. Actual review presents the main groups of physiologically active primary and secondary metabolites in the fruiting bodies of chanterelle such as indole and phenolic compounds, carbohydrates, fatty acids, proteins, free amino acids, sterols, carotenoids, enzymes, vitamins and elements with biological activity. The presence of these compounds and elements conditions the nutrient and therapeutic activity of chanterelle, e.g., immunomodulatory, anti-inflammatory, antioxidant, anti-viral, antimicrobial and antigenotoxic properties.

**Keywords**: *Cantharellus cibarius*, edible mushrooms, indole compounds, phenolic compounds, nutrition, antioxidant properties

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Table 1. Main groups of physiologically active metabolites and their contents in fruiting bodies of *Cantharellus cibarius* from natural states.

<table>
<thead>
<tr>
<th>Groups of identified compounds</th>
<th>Compound name</th>
<th>Content</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indole compounds</td>
<td>L-Tryptophan</td>
<td>0.02 mg/100 g d.w.</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>5-Hydroxytryptophan</td>
<td>0.01 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5-Methyltryptophan</td>
<td>0.68 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tryptamine</td>
<td>0.02 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Serotonin</td>
<td>17.61 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
<td>0.11 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indole</td>
<td>0.02 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Indoleacetonitrile</td>
<td>0.02 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kynurenine sulfate</td>
<td>3.62 mg/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td>Phenolic compounds</td>
<td>Protocatechuic acid</td>
<td>42.79 µg/g d.w.</td>
<td>42; 40*; 1**</td>
</tr>
<tr>
<td></td>
<td>1-Hydroxybenzoic acid</td>
<td>15.68 µg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vanillic acid</td>
<td>3.32 mg/kg d.w.*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sinapic acid</td>
<td>3.04 mg/kg d.w.*</td>
<td></td>
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<tr>
<td></td>
<td>Caffeic acid</td>
<td>16.34 µg/g d.w.</td>
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<tr>
<td></td>
<td>Ferulic acid</td>
<td>10.38 µg/g d.w.</td>
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<tr>
<td></td>
<td>Gallic acid</td>
<td>161.83 µg/g d.w.</td>
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<tr>
<td></td>
<td>Homogentisic acid</td>
<td>316.76 µg/g d.w.</td>
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<tr>
<td></td>
<td>Pyrogallol</td>
<td>91.09 µg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Myricetin</td>
<td>23.27 µg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>5.82 µg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cinnamic acid</td>
<td>1.29 mg/kg d.w.*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.97 mg/kg d.w.**</td>
<td></td>
</tr>
<tr>
<td>Organic acids</td>
<td>Oxalic acid</td>
<td>1.31 mg/g d.w.</td>
<td>44</td>
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<td></td>
<td>Malic acid</td>
<td>38.72 mg/g d.w.</td>
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<td></td>
<td>Citric acid</td>
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<td></td>
<td>Fumaric acid</td>
<td>1.63 mg/g d.w.</td>
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<td>Fatty acids</td>
<td>Pyrogallol</td>
<td>91.09 µg/g d.w.</td>
<td>44</td>
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<tr>
<td></td>
<td>Myricetin</td>
<td>23.27 µg/g d.w.</td>
<td></td>
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<tr>
<td></td>
<td>Catechin</td>
<td>5.82 µg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Palmitoleic acid</td>
<td>0.324 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Palmitic acid</td>
<td>15.660 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Heptadecanoic acid</td>
<td>0.504 mg/kg d.w.</td>
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</tr>
<tr>
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<td>Stearic acid</td>
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<tr>
<td></td>
<td>Oleic acid</td>
<td>148.16 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Linoleic acid</td>
<td>654.706 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Cis-11,14-eicosadienoic acid</td>
<td>0.088 mg/kg d.w.</td>
<td></td>
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<tr>
<td></td>
<td>Cis-8,11,14-eicosatrienoic acid</td>
<td>0.088 mg/kg d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Arachidonic acid</td>
<td>0.085 mg/kg d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Behenic acid</td>
<td>0.320 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Tricosanoic acid</td>
<td>0.061 mg/kg d.w.</td>
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<tr>
<td></td>
<td>Lignoceric acid</td>
<td>0.498 mg/kg d.w.</td>
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</table>
down from generation to generation for centuries. However, people harvested fungi only by considering appearance, taste or smell, because no nutritional or medicinal properties were considered. Therefore, mushrooms were for some time treated as a worthless and stodgy ingredient for some dishes, albeit a tasty one. In recent years, people have again become interested in mushrooms, because of

<table>
<thead>
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<th>Groups of identified compounds</th>
<th>Compound name</th>
<th>Content</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Amino acids &amp; 5’-nucleotides</td>
<td>Alanine</td>
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<td>Arginine</td>
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<td></td>
<td>Aspartic acid</td>
<td>0.06 mg/g d.w.</td>
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<tr>
<td></td>
<td>Cysteine</td>
<td>1.99 mg/g d.w.</td>
<td></td>
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<tr>
<td></td>
<td>Glutamic acid</td>
<td>29.99 mg/g d.w.</td>
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<tr>
<td></td>
<td>Glycine</td>
<td>0.13 mg/g d.w.</td>
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<td></td>
<td>Histidine</td>
<td>3.15 mg/g d.w.</td>
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<td></td>
<td>Leucine</td>
<td>0.21 mg/g d.w.</td>
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<tr>
<td></td>
<td>Lysine</td>
<td>5.74 mg/g d.w.</td>
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<td>Methionine</td>
<td>0.41 mg/g d.w.</td>
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<td></td>
<td>Serine</td>
<td>0.18 mg/g d.w.</td>
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<td>Threonine</td>
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<td></td>
<td>Phenylalanine</td>
<td>0.06 mg/g d.w.</td>
<td></td>
</tr>
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<td></td>
<td>Valine</td>
<td>1.34 mg/g d.w.</td>
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<td></td>
<td>Tryptophan</td>
<td>0.02 mg/g d.w.</td>
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<tr>
<td></td>
<td>5’-GMP</td>
<td>0.21 mg/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5’-AMP</td>
<td>0.41 mg/g d.w.</td>
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<tr>
<td></td>
<td>5’-CMP</td>
<td>0.09 mg/g d.w.</td>
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<tr>
<td></td>
<td>5’-IMP</td>
<td>0.03 mg/g d.w.</td>
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<tr>
<td></td>
<td>5’-UMP</td>
<td>0.75 mg/g d.w.</td>
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<tr>
<td></td>
<td>5’-XMP</td>
<td>0.14 mg/g d.w.</td>
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<tr>
<td>Carbohydrates</td>
<td>Mannitol</td>
<td>8.56 g/100 g d.w.</td>
<td></td>
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<tr>
<td></td>
<td>Trehalose</td>
<td>6.68 g/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mannose</td>
<td>8.56 g/100 g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>7.98 g/100 g d.w.</td>
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<tr>
<td>Carotenoids</td>
<td>β-Carotene</td>
<td>5.77 µg/g d.w.</td>
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<td></td>
<td>Lycopene</td>
<td>1.95 µg/g d.w.</td>
<td></td>
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<tr>
<td>Enzymes</td>
<td>Tyrosinase</td>
<td>0.09 µkat/g d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mn-dependent peroxidase</td>
<td>0.005 µkat/g d.w.</td>
<td></td>
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<td>Sterols</td>
<td>Ergosterol</td>
<td>24.7 mg/100 g f.w.</td>
<td></td>
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<td></td>
<td>Ergosta-7,22-dienol</td>
<td>0.4 mg/100 g f.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ergosta-5,7-dienol</td>
<td>0.4 mg/100 g f.w.</td>
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<tr>
<td></td>
<td>Ergocalciferol</td>
<td>10.7 mg/100 g f.w.</td>
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<tr>
<td></td>
<td>Ergosta-7-enol</td>
<td>0.2 mg/100 g f.w.</td>
<td></td>
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<tr>
<td>Tocopherols</td>
<td>α-Tocopherol</td>
<td>13.40 mg/g* d.w.</td>
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<tr>
<td></td>
<td>β-Tocopherol</td>
<td>2.87 mg/g* d.w.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ-Tocopherol</td>
<td>0.03 µg/g d.w.</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Cont.
the wealth of research which has confirmed the nutritional and medicinal value of fungi. Wild edible mushrooms are widely consumed in Asia, Western Europe and Central America. It has been estimated that 150,000 – 200,000 metric tons of the genus chanterelle alone are eaten annually worldwide (5). In 2012, in Poland, 5943 tons of wild edible mushrooms were commercially harvested, of which 3980 tons were C. cibarius (6). Chanterelle is a firm and durable mushroom, resistant to bacteria and worms, due to the dense structure and high concentration of the ergocalciferol and hydrophobic compounds (7, 8). The chemical composition of chanterelle depends on the place of origin, conservation procedures, environmental conditions etc. Chanterelle contains a great number of carbohydrates and proteins and a low amount of fat. This mushroom harvested in Croatia contains 31.91 g/100 g carbohydrates, 2.9 g/100 g lipids, 8.8 g/100 g ash, and the value of energy is 118 kJ/100 g of fresh fruiting bodies. C. cibarius from Greece contain fewer proteins (21.57 g/100 g dry weight (d.w.)), more fat (2.88 g/100 g d.w.) and more sugar (66.07 g/100 g d.w.) than chanterelles from Croatia (9, 10). The main groups of physiologically active primary and secondary metabolites present in the fruiting bodies of chanterelle are presented in Table 1.

Proteins and free amino acids
Crude proteins are one of the main components of chanterelle. The amount of these compounds is ranged to 53.7%, but the content of free amino acids is low - about 1% of d.w. Croatian researchers have investigated the fact that chanterelle fruiting bodies contain free amino acids such as alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, leucine, lysine, methionine, serine, threonine, phenylalanine, valine and tryptophan. The highest concentrations have been noted for glutamic acid (29.99 mg/g d.w.). From among the examined species (Agaricus campestris, Boletus edulis, Calocybe gambosa, Craterellus cornucopioides, Entoloma clypeatum, Flammulina velutipes, Macrolepiota procera, Morchella elata, Pleurotus ostreatus) chanterelle contains the greatest amount of lysine and threonine (5.74 and 8.98 mg/g d.w., respectively). Amino acids can be divided into four groups, connected with taste characteristics. Aspartic and glutamic acids are classified into the monosodium glutamate-like group. Alanine, glycine, serine, threonine are sweet taste amino acids, The third group – bitter taste amino acids – includes arginine, phenylalanine, histidine, leucine, isoleucine, methionine, and valine. Lysine and tyrosine are present in tasteless mushrooms. According to this classification, in chanterelle fruiting bodies the major group is the monosodium glutamate-like amino acids (30.05 mg/g d.w.). From among the examined species chanterelle possesses the highest concentration of bitter amino acids (10.15 mg/g d.w.). The meaty taste of mushrooms is attributable to the presence of 5'-nucleotides, mainly 5'-GMP. C. cibarius includes 0.41 mg/g d.w. of 5'-AMP, 0.09 mg/g d.w. of 5'-CMP, 0.21 mg/g d.w. of 5'-GMP, 0.03 mg/g d.w. of 5'-IMP, 0.75 mg/g d.w. of 5'-UMP and 0.14 mg/g d.w. of 5'-XMP. The umami taste of fungi may be increased due to the synergistic effect of the flavor of 5'-GMP and monosodium glutamate-like amino acids (9).

Carbohydrates
Carbohydrates compose 31.9% of the fruiting body dry mass. Chanterelle contains mannitol (8.56 g/100 g), trehalose (6.68 g/100 g), mannose (8.56 g/100 g), and glucose (7.98 g/100 g) (5). Mannitol is a naturally occurring polyol found in vegetables, fruits, mushrooms and in marine algae. It can be synthesized with lactic acid bacteria, fungi and yeast from fructose or glucose. In medicine, mannitol is used as a powerful osmotic diuretic. It reduces intracranial pressure, prevents oliguria and temporarily increases the passage of the drugs through the blood-brain barrier in the treatment of life-threatening diseases (11). Polysaccharides, belonging to the most important components comprise: chitin, chitosans. Chanterelle polysaccharides as other carbohydrates of Basidiomycota species (β-glucans) possess multidirectional antioxidant, anticancer activity (12, 13). They inhibit DNA damage, decrease the concentration of carcinogens and suppress their activation and the development of neoplastic cells by binding free radicals, immune system stimulation or induction of apoptosis. Because of their immunomodulating activity, mushrooms polysaccharides are also used to treat infectious diseases. Moreover, these compounds also possess antioxidant and antimicrobial activities. They are used to regulate and prevent hyperglycemia and hypercholesterolemia. The immunostimulating properties of mushroom polysaccharides consist of mitogenic activity, increased proliferation, differentiation and maturation of immune system cells and activation of stem cells in the process of hematopoiesis. Mushroom β-glucans are used in the treatment of dermatitis and in creams to protect the skin from UV radiation. β-Glucans, chitin, and various heteropolysaccharides may be classified as dietetic fibers. The main role of this group of com-
Fatty acids

PUFA (polyunsaturated fatty acids) intake is relatively lower than MUFA (monounsaturated fatty acids) and SFA (saturated fatty acids), even though PUFA have greater biological properties. PUFA are the crucial element of cell membranes. They maintain membrane fluidity and modulate cell function. Moreover, PUFA are the precursors of eicosanoids and lipid signaling molecules in human cells such as cardiac, muscle, nerve and immune cells. Prostaglandins, which belong to the group of eicosanoids, regulate inter alia cell growth, neurogenesis, synaptogenesis, blood pressure, and immune and inflammatory responses. PUFA can be consumed or synthesized endogenously from their 18C precursor. Ingested PUFA are better absorbed from the human gastrointestinal tract as a food ingredient than a formulated preparation. PUFA include three groups: n-3, n-6 and n-9 fatty acids. N-3 fatty-acids, synthesized from a linolenic acid, are the main compounds of animal oils and n-6, synthesized from linoleic acid, are the major ingredients in vegetable oils (15). Chanterelle contains lauric, myristic, pentadecanoic, palmitic, palmitoleic, heptadecanoic, stearic, oleic, linoleic, cis-11,14-eicosadienoic, cis-8,11,14-eicosatrienoic, arachidonic, behenic, tricosanoic, and lignoceric acids. The preponderant fatty acids are linoleic acid (654.706 mg/kg d.w.) and oleic acid (148.168 mg/kg d.w.). Chanterelle contains more saturated fatty acids (926.953 mg/kg d.w.) than polyunsaturated fatty acids (655.176 mg/kg d.w.) or monounsaturated fatty acids (148.493 mg/kg d.w.) (16). Fatty acids exhibit significant antifungal and antimicrobial properties. Compared to SFA, PUFA and MUFA are more potent fungicides. Acetylenic acids (a group of fatty acids containing one or more triple bonds) possess strong biological properties - antibacterial, antiviral, antifungal, antiparasitic, pesticidal and antitumor. These acids occur in plants, mushrooms, microbes, and marine organisms (17). Acetylenic acid (10E,14Z)-9-oxygenatedeca-10,14-dien-12-ynoic acid), isolated from methanolic extracts of C. cibarius, exhibits a significant transcriptional activity towards peroxisome proliferator-activated receptor γ that participates in the regulation of glucose and lipid metabolisms, cell growth and inflammatory processes. Isolated acetylenic acid does not modify the expression of stearoyl-CoA desaturase-1 gene, but it does reduce the expression of two glucoseoxygen genes, glucose 6-phosphatase and phosphoenolpyruvate carboxikinase, similar to the effect of troglitazone (18).

Vitamins

The fresh fruiting bodies of chanterelle contain ergosterol (24.7 mg/100 g f.w. (fresh weight)), ergosta-7,22-dienol (0.4 mg/100 g f.w.), ergosta-5,7-dienol (0.4 mg/100 g f.w.), and ergosta-7-enol (0.2 mg/100 g f.w.). Moreover, vitamin D₃ has also been identified (10.7 mg/100 g f.w.). Canned chanterelle contains less ergosterol (10.6 mg/100 g f.w.), but more of other sterols: ergosta-7,22-dienol (0.7 mg/100 g f.w.), ergosta-5,7-dienol (1.0 mg/100 g f.w.), ergosta-7-enol (1.6 mg/100 g f.w.) and more vitamin D₃ (12.1 mg/100 g f.w.). There has been research into whether the amount of vitamin D₃ can be increased by UV irradiation (19). The amount of 30–50 g fresh or canned chanterelle is sufficient to meet the daily nutrition for vitamin D (20). Vitamin D₃ can be synthesized endogenously (by conversion of 7-dehydrocholesterol during exposure to sunlight or UV radiation) or delivered with food or dietary supplements. Vitamin D possesses strong biological activity and prevents colon cancer, diabetes (type 1 and 2), glucose intolerance, hypertension, osteoporosis and multiple sclerosis (21). Ergocalciferol (vitamin D₂) is a scarce form of vitamin D in nature. It can be synthesized in the human body from ergosterol. Chanterelle fresh fruiting bodies are a good source of vitamin D₂ – they contain 14.2 µg/100 g f.w. Dried fruiting bodies contain 0.12 – 6.3 µg/g d.w. of ergocalciferol (average: 1.43 µg/g) after 2 – 6 years of storage. UV radiation affects the content of vitamin D₂ in the fruiting bodies (8). It was found that chanterelle is rich in vitamins A and E. Vitamin E occurs naturally in eight forms: α-, β-, γ-, and δ-tocopherol and α-, β-, γ, and δ-tocotrienol (22, 23). Among these molecules α-tocopherol shows the highest activity. Vitamin E has anti-inflammatory, antioxidant and anticancer activities. Moreover, it prevents DNA damage and decreases the risk of cardiovascular disease (22). Chanterelle is a species distinguished from other mushroom species by the greatest, comparable with baking yeasts, content of vitamin B complex: B₆, B₇, B₉, H³ and C (0.40 mg/g) (24, 25).

Pigments

Carotenoids, poliene pigments with a yellow, orange or red tint, are produced by plants and some
bacteria and fungi. Animals must receive these compounds in their diet. In humans, β-carotene, α-carotene, and β-cryptoxanthin can be converted to retinal, which is responsible for crepuscular vision. Moreover, it is also required for normal epithelium function and cell division. In the eye it absorbs damaging blue and near-ultraviolet light to protect the part of the eye with the sharpest vision. Fungal carotenoids protect fruiting bodies from UV damage. All carotenoids act as antioxidants. Chanterelle contains β-carotene, α-carotene, β-carotene and lycopene (24, 25). The major compounds are β-carotene (5.77 µg/g) and lycopene (1.95 µg/g) (25). Chanterelle antioxidants prolong their lifetime compared with other Basidiomycota species. On the basis of its high carotenoid content, C. cibarius is used as a natural source of these pigments (25). The fruiting bodies of C. cibarius are source of flavonoids, and total content of these compounds amounted to 0.47 mg/g (25).

**Enzymes**

Chanterelle contains active tyrosinase (0.09 µkat/g d.w.) and Mn-dependent peroxidase (0.005 µkat/g d.w.) (26). Moreover, in its fruiting bodies also contains laccase – ligninolytic enzyme. This has immunomodulating, antiproliferative and HIV-1 reverse transcriptase-inhibiting activities (27). The smell of chanterelle is because of the presence of 1-octen-3-ol. This substance is formed as a result of free linoleic acid oxidation in a process which is catalyzed by oxidoreductases. Especially during the drying process, this reaction is intensified (28).

**Non-hallucinogenic indole compounds**

Indole compounds can be divided into two groups: hallucinogenic and non-hallucinogenic. Mushrooms could accumulate both hallucinogenic indoles, for instance psilocybin, psilocin and baeocystin, and non-hallucinogenic, such as tryptamine, serotonin and tryptophan (29). It has been reported that methanolic, ethanolic and aqueous mushroom extracts inhibit lipid peroxidation, and destroy peroxides and hydroxyl radicals. These antioxidative properties are increased by the presence of vitamin C, phenolic compounds, amino acids, selenoproteins and indole compounds. In vitro examination has shown that melatonin and other indole derivatives (N-acetylserotonin, 6-methoxytryptamine) reduce lipid peroxidation in a dose dependent manner (30). Muszyńska investigated whether either extracts from chanterelle or in vitro cultures, derived from it, contain similar indole compounds: serotonin, melatonin, L-tryptophan, 5-hydroxytryptophan, 5-methyltryptophan, indole, indole-3-acetonitrile and kynurenine sulfate. Additionally, fruiting bodies also accumulate tryptamine unlike mycelial cultures. The highest concentration either in fruiting bodies or in in vitro culture was noted for serotonin and kynurenine sulfate. However, mycelial culture accumulates more serotonin (20.49 mg/100 g d.w.) and kynurenine sulfate (35.34 mg/100 g d.w.). To compare, the fruiting bodies contain 17.61 mg/100 g d.w. and 3.62 mg/100 g d.w. of these indole derivatives, respectively. It is worth noting that mycelial culture accumulates 1000 times more 5-hydroxytryptophan than fruiting bodies (12.52 mg/100 g d.w. and 0.01 mg/100 g d.w., respectively) (31, 32). Tryptophan is used as an element in protein synthesis and as a precursor of serotonin, melatonin, kynurenic acid, anthranilic acid and nicotinamide. Interestingly, nicotinamide (also called niacin or vitamin B₃) is only synthesized in our body from tryptophan. However, the amount of niacin produced is not always sufficient to meet the daily requirement for this vitamin in humans (33). Tryptophan can also be converted to tryptamine with tryptophan decarboxylase. Tryptamine is a precursor of phytohormones such as indole-3-acetic acid and mammal hormones such as serotonin and melatonin (34). Melatonin, another tryptophan derivate, is responsible for daily and seasonal activity, regulation of body temperature and mammalian reproduction. Moreover, it is also a potent antioxidant and anti-apoptotic agent, known as amphiphilic substance in comparison with other antioxidants, which are rather lipophilic or hydrophilic. Because of this property, melatonin can easily cross the cell membrane and reduce oxidative damage inside the cell. Visible effects of its protective influence include increased sperm viability, oocyte maturation and embryo development (35). Serotonin (5-hydroxytryptamine) is a neurotransmitter synthesized in the central nervous system. Serotonin is used to treat depression, schizophrenia, epilepsy and even eating disorders (36). Kynurenic acid is a final product of the tryptophan metabolic pathway in the mammalian brain. It is an endogenous non-selective antagonist of all subtypes of glutamate receptors: N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and kainate receptors, which are involved in motor activity, cognition, learning, memory and transmission of pain signals. Kynurenic acid, which is produced peripherally, inhibits homocysteine and leads to proliferation and migration of endothelial cells. The potential anti-atherogenic activity of kynurenic acid, its common occurrence in food and herbal preparations, the ease
of absorption from the gastrointestinal tract, the lack of metabolism in the human body and especially the low toxicity indicates that kynurenic acid is safe and easy to use in a wide range of patients with increased risk of cardiovascular disease (38).

**Phenolic compounds**

Phenolics are aromatic hydroxylated compounds which possess wide biological activity. In this group, phenolic acids have antioxidant, anti-inflammatory, antimicrobial, hypoglycemic and immunostimulating activity. Moreover, they decrease the risk of cardiovascular diseases and cancer. Phenolic acids protect plant and fungi cells from UV, insect and microbial damage. Potent antioxidant activity arises from synergic or antagonistic effects between individual polyphenols. Moreover, it is used to assure the stability of food products (39). Phenolic compounds are the most abundant antioxidants in the human diet. Their daily intake is estimated at 1 g, while the daily intake of phenolic acids is about one third of this amount and they are considered to be the most valuable antioxidants (40, 41). Chanterelle harvested in Poland contains protocatechuic, p-hydroxybenzoic, vanillic, sinapic and cinnamic acids and their content ranges from 1.29 to 3.32 mg/kg d.w. The highest concentration is noted for vanillic acid (3.32 mg/kg d.w.). This compound has only been detected in chanterelle, unlike in the remaining examined species (B. badius, B. edulis, A. mellea, L. deliciosus, P. ostreatus) (42). Barros reported in this species only cinnamic acid (14.97 mg/kg d.w.) (43). Chanterelle from Spain contains caffeic acid (16.34 mg/g d.w.), ferulic acid (10.38 mg/g d.w.), gallic acid (161.83 mg/g d.w.), p-hydroxybenzoic acid (15.68 mg/g d.w.), homogenetisic acid (316.76 mg/g d.w.), protocatechuic acid (42.79 mg/g d.w.), pyrogallol (91.09 mg/g d.w.), myricetin (23.27 mg/g d.w.) and catechin (5.82 mg/g d.w.). Among the examined species, chanterelle possesses the highest content of caffeic acid (respectively, A. bisporus 15.54 mg/g d.w., B. edulis 15.09 mg/g d.w., C. gambosa 14.92 mg/g d.w., H. marzuolus 14.59 mg/g d.w. and L. deliciosus 15.51 mg/g d.w.). Catechin has only been detected in chanterelle and A. bisporus, although the concentration of this compound is 11.4 times higher in chanterelle than in A. bisporus. Total phenolics and flavonoids content have been also examined. Interestingly, the decline in oxidation yield does not correlate either with the flavonoid content or with the total phenolic amount, and this may be a result of the different antioxidant activities of the individual phenolic acids. As an example, chanterelle contains more caffeic acid and catechin than other species, and probably these compounds possess higher antioxidant activity than other detected polyphenols. (42, 44). Preservation procedures alter the nutritional value of mushrooms. Dried and frozen chanterelle contains 3-O-cafeoylquinic acid, 4-O-cafeoylquinic acid, 5-O-cafeoylquinic acid, caffeic acid, p-coumaric acid and rutin. Samples preserved in olive oil contain examined compounds: hydroxytyrosol, tyrosol, rutin, caffeic acid, p-coumaric acid, luteolin and apigenin and showed the highest total concentration of them (994.4 mg/kg d.w.). Tyrosol and hydroxytyrosol are the major compounds in samples preserved in vinegar (around 80% and 13%, respectively) and in olive oil (both around 40%). Chanterelle conserved in vinegar contains only hydroxytyrosol, tyrosol, rutin. Rutin is the only common component for all examined samples (44, 45).

**Organic acids**

Wild chanterelle harvested in Northeast Portugal and commercial sporocarps from Portugal supermarkets were analyzed for the content of organic acid. Citric, ascorbic, malic, fumaric, and shikimic acids are found in conserved fruiting bodies of chanterelle. Malic acid is the predominant compound in samples which are frozen, dehydrated or conserved in olive oil. Frozen samples contain higher amounts of this substance than other ones. In fruiting bodies conserved in vinegar ascorbic acid was the major compound (45). Oxalic acid, malic acid, fumaric acid, were detected in wild and commercial fruiting bodies and contents ranged from 1.31 to 59 mg/g d.w. Citric acid was examined in chanterelle of natural origin (12.02 mg/g d.w.). Total content of identified organic acids was higher in commercial fruiting bodies of this species (64.71 mg/g d.w.) than in fruiting bodies from nature (53.68 mg/g d.w.) (46).

**Elements**

Stegnar reported that fruiting bodies of Basidiomycota species are able to accumulate Hg (47). About 20 years later, the accumulation of other elements in Armillaria mellea fruiting bodies was extensively investigated by Falandysz (48). More recently, Kalač reported that edible mushrooms are able to accumulate elements in their fruiting bodies (49).

Iron concentrations in chanterelle fruiting bodies and in vitro cultures vary insignificantly (respectively 435.7 and 457.9 µg/g d.w.). Mainly, fruiting bodies accumulate micronutrients better than
mycelium from in vitro cultures. For instance, the concentration of magnesium is twice as high in fruiting bodies (1004.1 µg/g d.w.) as in mycelium (541.8 µg/g d.w.); this is similar for copper (43.57 µg/g d.w. and 12.47 µg/g d.w., respectively). However, zinc exhibited an inverse tendency – 131.9 µg/g d.w. is accumulated by in vitro cultures and 95.5 µg/g d.w. by fruiting bodies. Nickel is accumulated in the lowest amount: 1.88 µg/g d.w. by fruiting bodies and 0.33 µg/g d.w. by in vitro cultures (50). Mushrooms accumulate more microelements than plants. However, they are also able to accumulate toxic metal ions in high concentrations. Chanterelle accumulates less Hg than Boletus edulis and Leccinum aurantiacum. The efficiency of Cu, Cd, Zn, Pb, Ni accumulation in chanterelle fruiting bodies is the lowest among examined species (51). Chanterelle contains selenium, which is a part of the glutathione peroxidase, that protects the organism from oxidative damage (7). Mleczek found that chanterelle also accumulates Al (40.9 mg/kg d.w.), Ba (1.9 mg/kg d.w.), Ca (68.0 mg/kg d.w.), Fe (91.8 mg/kg d.w.), K (21017.9 mg/kg d.w.), Mg (309.3 mg/kg d.w.), Mn (35.7 mg/kg d.w.) and Na (73.9 mg/kg d.w.). The bioaccumulation factor is the highest for K (210.2 mg/kg d.w.) and Mg (3.1 mg/kg d.w.) (52).

**BIOLOGICAL ACTIVITY OF EXTRACTS FROM FRUITING BODIES OF YELLOW CHANTERELLE**

As a result of the presence of a variety of compounds with therapeutic properties, mushrooms have both nutritive and also medical properties. They can prevent hypertension, hypercholesterolemia and cancer. Most of them possess immunomodulatory, anti-inflammatory, antioxidative, antiviral and antimicrobial activities (53).

Although *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans* are not sensitive to water extracts of chanterelle, the methanolic extract of these fungi showed slight antimicrobial activity against *S. aureus*. These differences may result from the oxidative reactions of the active compound such as phenolic acids during extraction procedures. Therefore, Santoyo et al. (54) added peroxidases and polyphenol oxidase inhibitors (potassium pyrosulphite and sodium azide) to the water extracts in order to examine the influence of the oxidative enzymes on the antimicrobial activity of water extracts. Results showed that the addition of both inhibitors in 0.1 M concentration significantly increases the antimicrobial potential of chanterelle water extract. Barros et al. (25) found that methanolic extracts of this species presented antimicrobial activity against *Bacillus cereus*, *Bacillus subtilis*, *S. aureus*. Ethyl acetate, acetone, chloroform and ethanol extracts of chanterelle were tested and it was found that these extracts revealed antimicrobial activity against some Gram (+) and Gram (-) bacteria, yeasts, filamentous fungi and the actinomycetes (55).

The primary oxidative stress-response pathway is the NF-kB pathway. NF-kB is normally present in the cytoplasm as a homo- or heterodimer. After phosphorylation, NF-kB translocates from the cytoplasm to the nucleus. Ergosterol, cerevisterol, tuberoside, β-sitosterol, glucoside, ergosterol peroxide, β-sitosterol and 7-dehydrostigmasterol and a cerebroside have been isolated from the n-hexane and ethyl acetate extracts of the fruiting bodies of chanterelle. These active metabolites inhibit NF-kB activation by inhibition of the NF-kB translocation from the cytoplasm to the nucleus (56).

Santoyo group of Mexican researchers examined the antigenotoxic activity of chanterelle aqueous extracts against damage induced by methyl methanesulfonate in human mononuclear cells. All protocols (previous, simultaneous and posterior) included 5 extracts in different concentrations [%]: 0.0125, 0.025, 0.05, 0.1 and 0.2, respectively. Aqueous extracts efficiently reduced DNA damage in simultaneous and previous treatment. The highest reduction of DNA damage was exhibited by 0.0125% extract from the previous protocol and the 0.2% extract from the simultaneous one. No dose dependent effect was observed (57).

Extracts from chanterelle fruiting bodies include lectins, which preferentially agglutinate human type A erythrocytes. However, the same lectins exhibit non-specific hemaggulination activity against rabbit erythrocytes. Lectins are carbohydrate-binding proteins, which are highly specific for sugar moieties. They have a non-immune origin and bind non-covalently and reversibly to specific carbohydrates on the cells (58).

Khalili et al. (59) examined whether methanolic extracts of chanterelle possess strong antihypoxic activity in hemic and circulatory hypoxia models. In hemic hypoxia, extracts at 300 mg/kg were able to keep mice alive for 9.94 ± 0.87 min and at 600 mg/kg for 10.07 ± 1.18 min in comparison to control groups (7.00 ± 0.63 min). The results of circulatory hypoxia showed prolonged survival time among treated mice to 15.18 ± 4.21 min (600 mg/kg extracts) and 13.57 ± 0.87 min (300 mg/kg extracts) compared with the control groups 9.84 ± 0.75 min. It was the first study on the antihypoxic properties of chanterelle extracts. Vamanu and Nita (60) reported
anti-inflammatory activity of this species extracts which were tested by inhibition of albumin denaturation and erythrocyte membrane stabilization. Phenolic compounds (homogentisic and gallic acids) and carotenoids (lycopene) were mainly responsible for the anti-inflammatory and antioxidant effects.

CONCLUSIONS

In conclusion, chanterelle possesses dietary and medicinal properties. The combinations of carbohydrates, amino acids, unsaturated fatty acids and vitamins revealed nutritional properties of this species. This fungus is also a highly valued source of carotenoids, tocopherols, vitamin C, flavonoids, sterols, phenolics, indole compounds and selenium. Some of these physiologically active compounds may be potentially used in dietary supplements or medicines. These compounds and elements are decisive for the antioxidant properties so they play a protective role against civilization diseases. Extracts of chanterelle presented immunomodulatory, anti-inflammatory, antiviral, antimicrobial and antigenotoxic activities.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES


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Therapeutic drug monitoring or therapeutic drug management

Therapeutic drug monitoring (TDM) refers to the quantitative measurement of therapeutic drugs in serum, plasma or other biological fluids. The purpose of TDM assessment is to provide health care professionals with information to assist in adjusting a patient’s drug regimen to reach an optimal drug concentration that will ensure the patient achieves the desired therapeutic response without adverse drug reactions or toxicity. TDM assumes that there is a relation between serum drug concentration and its pharmacological effect. Indications for therapeutic drug monitoring include: lack of therapeutic effect or occurrence of adverse events, narrow therapeutic window, lack of possibility of optimizing drug dose based on clinical observation or laboratory results, substantial interindividual differences in pharmacokinetics, i.e., drug absorption, distribution, metabolism and excretion according to age, genotype, etc., comorbidities like renal and hepatic failure influencing drug excretion and metabolism, gastrointestinal disorders influencing drug absorption, non-linear pharmacokinetics, drug-drug interactions, difficulty in interpreting signs and symptoms of toxicity or therapeutic failure, potential patient compliance problems. Drugs that are commonly analyzed by therapeutic drug monitoring comprise: aminoglycoside antibiotics (gentamicin), vancomycin, antiepileptics (such as carbamazepine, phenytoin and valproic acid), digoxin, antiarrhythmics (such as procainamide and lidocaine), psychoactive drugs (lithium, tricyclic antidepressants), theophylline, immunosuppressants (cyclosporine, tacrolimus), methotrexate (1). For these drugs clinical experience or clinical trials should have shown that TDM improved outcome in the general or special populations as it is stated in the definition by the International Association for Therapeutic Drug Monitoring and Clinical Toxicology (ATDMCT) (2).

Economic considerations in therapeutic drug monitoring/management

It needs to be stressed out that TDM is not a simple analysis of a single drug concentration in the blood but it also encompasses interpretation of the value measured with the use of pharmacokinetic modelling, drawing appropriate conclusions about the result and advising the clinician who ordered the test how to modify drug dose or dosing interval. Therapeutic drug monitoring service can be provided by adequately trained health care professionals, either by clinical pharmacists or clinical pharmacol-
ogists. The Therapeutic Drug Management and Toxicology Division of the American Association for Clinical Chemistry advocates to replace the word “monitoring” with “management” in all references to TDM in order to emphasize the purpose of the actual monitoring of drug concentrations in biological fluids. “Management” implies that the laboratory measurement is an essential part of the treatment of the patient, whereas “monitoring” is focused on the analytical process, without reference to the clinical implications (3).

In times of increasing financial pressure for hospitals and their budgetary constraints, cost-effectiveness analyses for TDM are required, just in order to justify the staffing of a clinical pharmacokinetic service (4). In 1966, Donabedian proposed the structure-process-outcome method for the assessment of quality of health care (5). Schumacher and Barr translated this method to TDM. The evaluation of the structure component applied to TDM includes adequacy of the TDM testing equipment and facilities, qualifications of clinical and laboratory staff, presence of a TDM service, supervision, and administrative organization. The process component for TDM involves procedures such as appropriate indications for ordering a serum drug level, timing of the sample collection, communication of results to clinicians and intervention based on the results. The outcome component comprises the effect of an intervention on the outcome of the patient and its impact on the health care system, what applied to TDM includes speed of recovery, number of adverse effects, morbidity, mortality and cost savings associated with a TDM service. In 1979, Bootman et al. demonstrated in a retrospective analysis of severely burn patients with Gram-negative sepsis that patients in whom gentamicin was dosed individually based on TDM were characterized by longer hospital stay, but on the other hand, by significantly lower mortality rate compared to those in whom gentamycin levels were not monitored. The extra costs of the TDM service were overweighted by the overall savings made as a result of reduced mortality (11).

Aim

The aim of this review was to summarize outcome and economic studies relevant to the use of therapeutic drug monitoring.

Aminoglycosides

Aminoglycoside antibiotics are important in the treatment of Gram-negative infections and as synergistic agents for the treatment of staphylococcal and streptococcal infections. They are still widely used despite the introduction of newer classes of antimicrobial agents. However, these agents have a narrow therapeutic index. Data suggest that a pharmacokinetic/pharmacodynamic relationship exists for some aspects of efficacy and toxicity of aminoglycosides. Peak serum drug concentrations and the ratio of peak serum drug concentration to minimum inhibitory concentration appear to correlate with their clinical efficacy. Tissue drug accumulation, as indicated by increasing trough concentrations, has been associated with ototoxicity and renal impairment. These relationships, and the wide inter- and intrapatient variability of pharmacokinetic parameters are the basis of therapeutic drug monitoring of aminoglycosides that aims to optimise therapy and avoid toxicity (8-10).

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TDM services have been shown to reduce aminoglycoside nephrotoxicity. In the study of Slaughter and Cappelletty, the costs of providing TDM averaged US$ 301.87 (1997 values) per patient and the cost of management of single nephrotoxicity was estimated at US$ 4583 (1997 values). In order for the costs of providing a TDM service to 100 patients (US$ 30,187) to be offset by cost savings due to decreasing nephrotoxicity, the service would need to be able to reduce nephrotoxicity by 6.6%, resulting in a saving of US$ 30,248. Therefore, TDM service may be cost justified only in populations where nephrotoxicity rate is high (e.g., > 15%) (12).

In the study of van Lent-Evers et al., active therapeutic drug monitoring service (ATM), assuming that the hospital pharmacist is involved in deciding the initial dose and dosing interval of an aminoglycoside for an individual patient, was compared with standard nonguided TDM. ATM group comprised 105 patients and nonguided TDM comprised 127 patients, including 48 and 68 persons, respectively, with an infection on admission. In ATM patients peak gentamicin concentrations were significantly higher compared to nonguided TDM patients (10.6 ± 2.9 mg/L, 7.6 ± 2.2 mg/L; p < 0.01). High trough gentamicin concentrations (more than 2 mg/L) were observed in 23% of nonguided TDM patients and in only 3% of ATM patients (p < 0.01). There was a trend toward higher mortality in nonguided TDM patients. Subgroup analysis of patients with an infection at admission showed significantly less mortality in the ATM group measured at day 28 after admission (p = 0.023). ATM reduced the length of hospital stay for all patients in the study (20.0 ± 1.4 days, 26.3 ± 2.9 days; p = 0.045) and for patients admitted with an infection (12.6 ± 0.8 days, 18.0 ± 1.4; p < 0.001) compared to the nonguided TDM group. There was significantly less nephrotoxicity in ATM patients compared with the nonguided TDM group (2.9%, 13.4%; p < 0.01). ATM was shown to save costs in comparison with the usual care with nonguided TDM. Total costs calculated as the sum of costs related to the number of days in the ward, intensive care unit costs, intervention and TDM costs and expressed in Dutch guilders [DFL], were lower for all patients (13.125 ± 9.267, 16.862 ± 17.721; p < 0.05) and for patients admitted with an infection (8.883 ± 3.778, 11.743 ± 7.437; p < 0.01). The ATM strategy provided better control of therapy because the a priori consultation facilitated consideration of physiologic and pathologic parameters with the intention of treating the patient and not the levels. Tailoring the dose and the interval to the patient’s need and adjusting the duration of the aminoglycoside course based on the patient’s response became feasible. This resulted in a decrease in mortality, length of hospital stay and reduction in the incidence of nephrotoxicity proving the cost-effectiveness of ATM. Subgroup analysis showed that it was even more beneficial for patients admitted to the hospital with a suspected or proven infection (13).

In a 1-year retrospective historically controlled study before and after the implementation of guidelines, Fonzo-Christe et al. evaluated their impact on dosing and TDM practice, blood sampling, and therapeutic concentrations of gentamicin in newborns. Guidelines recommending once-daily dosing (ODD) or extended-interval dosing (EID) and trough concentration measurement were developed and implemented as clinical decision support in the computerized prescriber order entry system in Geneva University Hospitals. After implementation of the guidelines, an ODD/EID regimen was almost exclusively used (97.7% versus 61.6%, p < 0.001), the percentage of peak concentrations (0.9% versus 17.2%, p < 0.001) and the number of blood samples per patient (87.1% having 0 or 1 concentration measured versus 48.0, p < 0.001) relevantly decreased. More than two-thirds of newborns had trough concentrations under 1 mg/L what was significantly higher percentage in comparison with about one-third before implementation (p < 0.001). Although cost-effectiveness analysis was not performed, better outcome and lower cost of TDM favor such proceedings (14).

Vancomycin

Like for aminoglycosides, there seems to be a relationship between vancomycin serum concentrations and both its efficacy and toxicity. A prospective cohort study was conducted to document differences in the outcome of vancomycin therapy in patients managed through a therapeutic drug monitoring (TDM) service (n = 61) and patients managed empirically (n = 55). Outcome measures were as follows: duration of therapy, total vancomycin dosage, infection site, concomitant antibiotics, body temperature, white blood cell counts, length of hospital stay and nephrotoxicity. On average, patients receiving TDM had less nephrotoxicity (7% vs. 24%), needed lower cumulative vancomycin dosages (of 5 g less) and had shorter hospital stays (38 vs. 44.5 days) in comparison with those who did not receive TDM (15).

In the study of Fernandez de Gatta et al., 70 immunocompromised febrile patients with hematologic malignancies were randomly assigned to
either a vancomycin therapeutic drug monitoring group \((n = 37)\) or to a control group \((n = 33)\). The rates of minor nephrotoxicity were 33.3\% and 13.5\% in the control and TDM group, and the rates of moderate nephrotoxicity were 9.1\% and 0\%, respectively. A decreased incidence of nephrotoxicity proves a real clinical benefit from the vancomycin monitoring program in this patient population. An incremental cost of $435 per case of vancomycin monitoring program in this patient population proves a real clinical benefit from the vancomycin therapy what makes it a cost-effective procedure again in this high-risk population (16).

Based on the data of the above mentioned studies Darko et al. performed a decision analysis to model the cost-effectiveness of pharmacokinetic dosage adjustment of vancomycin to prevent nephrotoxicity. It was assumed that at least two - one peak and one trough - vancomycin serum concentrations would be obtained during therapy. The frequency of trough concentrations less than or equal to 10 mg/L and greater than 10 mg/L was calculated. The probabilities of nephrotoxicity associated with vancomycin trough concentrations data were derived from recent clinical trials. The mean cost of treating nephrotoxicity was SUS 11,233. The cost of preventing one vancomycin-associated nephrotoxic episode with TDM was calculated as SUS 25,166. The subgroup analysis revealed a cost of 8,363 dollars/nephrotic episode prevented was calculated for the TDM for vancomycin therapy what makes it a cost-effective procedure again in this high-risk population (16).

Of the same concern may be the potential for treatment failure because of underdosing to avoid the risk of toxicity. Thirty one episodes of Gram-positive peritonen dialysis patients were reviewed in the study by Mulhern et al. in an attempt to identify the risk factors for peritonitis relapse. All patients were treated with 4 weekly doses of intravenous vancomycin. Nine peritonitis episodes complicated by a relapse were identified. Peritonitis episodes preceding a relapse were then compared to relapse-free episodes and there was drawn a conclusion that a suboptimal trough serum vancomycin level (cumulative 4-week trough serum vancomycin level < 12 mg/L or an initial 7-day trough serum level < 9 mg/L) was the only clinical parameter that identified peritonitis episodes at risk for relapse (18).

### Antiepileptic drugs

Rane et al. have demonstrated that therapeutic drug monitoring consisting of a minimum of two drug estimations per year offers significant benefit in terms of better seizure control, fewer adverse events and greater chances of remission in adult patients with generalized tonic-clonic epilepsy. A retrospective, post hoc pharmacoeconomic analysis of a cohort of patients who had undergone TDM and controls matched for age, disease, duration of drug therapy and duration of epilepsy clinic attendance was carried out. At interview one year after treatment, in the study group 11/25 patients had achieved complete seizure control, 10/25 patients had 50% reduction in seizure frequency, while 4/25 patients still had uncontrolled epilepsy whereas in control group 2/25 patients had reached complete control, 11/25 patients had 50% reduction in seizure frequency, while 12/25 patients still had uncontrolled epilepsy. There was a significant difference in seizure control between groups. The incidence of adverse events was also significantly lower in the TDM group (2/25 vs. 10/25; \(p < 0.05\)). TDM patients also had a better social status: at the time of interview 19 vs. 12 patients were earning, 15 vs. 7 patients were married and had children. The cost per sample analyzed to the hospital was 147 Indian rupees. The cost to the hospital per seizure prevented was estimated 22.35 rupees while the cost to the patient prevented was 22.35 rupees while the cost to the patient per seizure prevented was 4.50 Indian rupees (19).

### Digoxin

A post-hoc analysis of the Digitalis Investigation Group trial was performed in order to assess variations in serum digoxin concentration and their association with mortality and hospitalization in patients with heart failure. The analysis was restricted to men with a left ventricular ejection fraction of 45% or less \((n = 3782)\). Patients randomly assigned to receive digoxin were divided into 3 groups based on serum digoxin concentration at 1 month \((0.5-0.8 \text{ ng/mL}, n = 572; 0.9-1.1 \text{ ng/mL}, n = 322; \text{and} \geq 1.2 \text{ ng/mL}, n = 277)\) and compared with patients assigned to receive placebo \((n = 2611)\). The primary end point of the Digitalis Investigation Group trial was all-cause mortality within 37 months of randomization. There was no difference in all-cause mortality among patients randomly assigned to placebo and patients assigned to digoxin who had serum digoxin concentrations assessed. However, patients with serum digoxin concentrations of 0.5 to 0.8 ng/mL had a 6.3\% (95\% CI, 2.1-
10.5%) lower mortality rate compared with patients receiving placebo, digoxin was not associated with a reduction in mortality among patients with serum digoxin concentrations of 0.9 to 1.1 ng/mL, whereas patients with serum digoxin concentrations of 1.2 ng/mL and higher had an 11.8% (95% CI, 5.7-18.0%) higher mortality rate than patients receiving placebo. Similarly, patients with higher serum digoxin concentrations had higher crude rates of all-cause hospitalization and patients with lower serum digoxin concentrations had lower crude rates of all-cause hospitalization than patients randomly assigned to placebo. This study strongly suggests that digoxin offers a survival advantage in a narrow therapeutic window and therefore TDM of digoxin may be warranted in men with heart failure and left ventricular dysfunction (20).

**Immunosuppressants**

Cyclosporine was the first immunosuppressive agent to receive serious attention regarding pharmacokinetics, pharmacodynamics and TDM. Most centers use two target ranges, one for initial therapy and the second for maintenance therapy thereafter. Because of the considerable variability in the bioavailability, metabolism, and excretion of cyclosporine in transplant recipients and because of the drug’s narrow therapeutic index, dosage individualization based on blood cyclosporine concentration is required to reduce the risk for either underdosage or toxicity. Nevertheless, there are no studies published that have formally looked at the cost-effectiveness of TDM for cyclosporine and other immunosuppressants. Because it has been clearly established that TDM increases the chance of one-year survival of transplanted kidney from 60% to 95%, a randomized study that investigates the cost-effectiveness of TDM vs. no monitoring in transplant patients would be ethically unacceptable (21).

However, prospective concentration-controlled studies performed with validated analytical methodology for cyclosporine and other immunosuppressants, examining the risk/benefit ratio for specific concentrations of these drugs in specific patient groups would be highly recommended. The vast majority of centers use a predose trough blood sample for cyclosporine analysis. It has been recently demonstrated that trough concentrations correlate poorly with the AUC (area under the curve), and thereby do not adequately reflect cyclosporine exposure, whereas improved correlation with clinical effects of the drug is achieved by evaluating its total exposure, i.e., AUC (22). A practical limitation of this approach is the necessity to collect several blood samples at defined time points. Adequate cyclosporine exposure during the first 4 hours after intake (AUC0-4) has been proven to correlate with freedom from rejection and toxicity and C2 (a 2-hour postdose cyclosporine level) has been determined to be the best single-timepoint predictor of the AUC0-4 (23).

The study of Shenoy et al. purposed to compare the safety, efficacy, and pharmacoeconomics of cyclosporine monitored by C2 levels and tacrolimus monitored by trough levels in de novo liver transplant recipients. After informed consent, 60 de novo liver transplant recipients were randomized in a 1:1 fashion to receive either tacrolimus (trough, 6-10 ng/mL) or cyclosporine (C2, 600-1200 ng/mL) and corticosteroids. The primary endpoint was the rate of acute rejection at 12 months. Incidence of infection, adverse events, and drug costs were secondary endpoints. Early acute rejection occurred in 27% of tacrolimus-treated patients and 23% of cyclosporine-treated patients (NS). Recurrent HCV occurred in 21% of tacrolimus-treated patients and 61% of cyclosporine-treated patients (p = 0.04). The incidence of new onset diabetes mellitus, requirement for antihypertensives and for cholesterol medications were similar between the groups. Annual calcineurin inhibitor costs were lower for cyclosporine (US$ 5432 ± 2091 vs. US$ 8291 ± 3948, p = 0.001), although total 1-year posttransplant drug costs were similar (US$ 17,214 ± 16,600 vs. US$ 15,151 ± 11,699, NS) (24).

**CONCLUSIONS**

Therapeutic drug monitoring service providing appropriate pharmacokinetic interpretation and recommendations to clinicians has already been proven either to be cost-effective or to improve outcome. The question of at least the same importance is how to increase the effectiveness of TDM, i.e., determining specific patient groups, developing reliable and easy to use assays, etc. In conclusion, it can be recapitulated that TDM of aminoglycosides is cost-effective, leads to a reduction of mortality, nephrotoxicity and length of hospital stay. TDM of vancomycin results in reduced nephrotoxicity and is cost-effective in selective patients populations, such as ICU patients, oncology patients and patients treated with other nephrotoxic drugs. TDM of classic antiepileptic drugs can offer significant benefit in terms of better seizure control and fewer side effects. TDM of digoxin may be useful in cardiac failure. Because of a shortage of donor organs, considerable interindividual variability and risk for
drug-drug interactions therapy with immunosuppressants must be guided by TDM.

REFERENCES

4. Touw D.J., Neef C., Thomson A.H., Vinks A.A. on behalf of the Cost-Effectiveness of Therapeutic Drug Monitoring Committee of the International Association for Therapeutic Drug Monitoring and Clinical Toxicology: Ther. Drug Monit. 27, 10 (2005).

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It is not without a reason that the 20th century was named a **century of separation science** (1). After more than a hundred years of the development of chromatographic techniques, analytical chemistry has gained a tool without which knowledge about the environment, food and natural products would have been much poorer. The opportunities created by chromatographic techniques are also used by professionals involved in pharmaceutical analysis, who often study samples characterized by complex composition. According to the most popular definition, **pharmaceutical analysis can be simply defined as analysis of a pharmaceutical compound or drugs.** This branch of analytical chemistry investigates new drugs, contaminants in pharmaceutical preparations, the pharmacokinetics and metabolism of drugs as well as toxicological and biotechnological topics (1, 2). The term pharmaceutical analysis also refers to the analysis of plant raw materials which are used in the production of pharmaceutical preparations and herbal drugs (3).

Although many published studies deal with the present-day position of chromatographic techniques in pharmaceutical analysis (1, 4, 5), there are no relevant publications about the historical development of these techniques. At the same time, it is noteworthy that chromatographic techniques have been used in pharmaceutical analysis since the interwar period. The aim of this work is to provide information about the development of chromatographic techniques...
used in pharmaceutical analysis. The sources of information were Polish pharmaceutical journals, i.e., „Kronika Farmaceutyczna”, „Farmacja Współczesna”, „Wiadomości Farmaceutyczne”, „Acta Poloniae Pharmaceutica”, „Farmacja Polska”, „Annales UMCS sectio DDD Pharmacia” and „Dissertationes Pharmaceuticae” that were available at the library of the Jagiellonian University Medical College and the University of Warsaw Library.

Two information databases, namely, Polish Medical Bibliography (The Central Medical Library, Warsaw) and the PubMed database (US National Library of Medicine National Institutes of Health, Bethesda) were also searched.

**Capillary analysis**

In retrospect, there is no doubt that chromatographic techniques have played a key role in the development of research in the field of pharmaceutical analysis. The first application of chromatography to pharmaceutical analysis can be traced back to the use of a so-called capillary analysis. The principles of this technique were presented in two textbooks authored by Professor Bronisław Koskowski, a member of the Faculty of Pharmacy, University of Warsaw. In the textbook titled „The science of preparing drugs and their forms” (1925), lectures on research methodology using capillary analysis can be found. The aforementioned reference was based on the mechanism described for paper chromatography, however, a typical mobile phase and the sample application spot were lacking. The process of chromatographic separation is a result of the flow of fluid sample through paper. The sample components move at different rates due to the differences in their affinity for the stationary phase. In the case of pharmaceutical analysis, the samples were mostly alcoholic or aqueous extracts of plant materials (6). In his second textbook, titled „Capillary analysis applied to assess the identity and goodness of pharmaceutical preparations” (1933), Koskowski presented the results of capillary analysis carried out for all tinctures that were supposed to be included in „Polish Pharmacopoeia II”. There were also colorful illustrations showing capillary images of the studied tinctures. All this information was supposed to serve as guidelines for routine testing performed in pharmaceutical laboratories (7).

In the 1930s, capillary analysis was used in several works dedicated to the qualitative evaluation of the composition of herbal tinctures. In 1932, Tadeusz Goettinger published the article titled „Detection of alkaloids in tinctures by capillary analysis” in „Wiadomości Farmaceutyczne” (8), while Ludmiła Świerczyńska published a paper titled „About the possible applications of capillary analyses to study magistral preparations” in „Farmacja Współczesna”. Both publications were based on studies conducted in the Gessner Pharmacy laboratory in Warsaw (9). In 1937, capillary analysis was criticized by Władysław Karaffa-Korbutt, a professor of the Stefan Batory University in Vilnius, in the paper titled „Capillary-luminescent analysis of pharmaceutical preparations”. Karaffa-Korbutt pointed out the shortcomings of capillary analysis and, at the same time, proposed the modification, i.e., the use of quartz lamp for analyzing the capillary images. The modified technique produced more characteristic and reproducible results (10).

In the postwar period, capillary analysis was replaced by paper chromatography. However, several publications employing capillary analysis and dealing with the subject of qualitative differentiation of tinctures obtained from the medicinal plants growing in various locations, harvested at different times, or obtained from various plant organs (11, 12) were still published.

**Column chromatography**

The technique of classical column chromatography and the possibility of its use in pharmaceutical analysis were described in 1949 in a review article titled „The chromatographic analysis (chromatography) and its applications” and authored by Jan Muszyński, a professor at the Faculty of Pharmacy, Medical University of Lodz and President of the Polish Pharmaceutical Society. After a detailed presentation of the mechanism of column chromatography, the latest achievements in the field of chromatography were described in the next section of the aforementioned paper. A so-called ultra-chromatography technique using the analytical quartz lamp was among the presented methods. Thus, it was possible to study colorless compounds passing through a chromatographic column that were characterized by their fluorescence under UV irradiation. Based on the data from foreign literature, the author described several types of products that could be studied with this technique, i.e., leaf pigments, aniline dyes, alkaloid raw materials (analyzed via ultra-chromatography), and the separation of antimony and bismuth salts (13, 14).

In 1952, a methodological work titled „Chromatographic and biological studies of food dyes with particular emphasis on erythrosine” was published in „Acta Poloniae Pharmaceutica”. In this publication, 5 out of 30 tested food colorants were determined by column chromatography employing
different adsorbents, i.e., aluminum oxide, talc and calcium carbonate (15). It should be emphasized that the aforementioned study was also the first work employing a chromatographic technique that had been published in „Acta Poloniae Pharmaceutica”.

In later years, column chromatography was used in pharmaceutical analysis rarely, among others as a quantitative analysis technique. The results were presented as so-called *elution diagrams* (16). Column chromatography was also used as a preparative technique, as well as an auxiliary tool for a new chromatographic techniques, such as paper chromatography and thin layer chromatography (17-19). In the 1990s, column chromatography was used in the Immobilized Metal Ion Affinity Chromatography to study biomolecules, i.e., peptides and proteins (20).

**Paper chromatography (PC)**

The first Polish publication employing paper chromatography was dedicated to the qualitative and quantitative identification of medicinal substances. It was published in „Acta Poloniae Pharmaceutica” in 1952 under the title „The chromatographic separation of B vitamins”. The use of chromatographic techniques in this work was motivated by the small amount of test material needed. The analysis was conducted by using different mobile phase compositions, and an attempt was made to perform the quantitative analysis as well. A method of sample preparation and the analysis of yeast and bovine blood has been described (21). In 1953, a review article dedicated to chromatographic techniques was published in „Farmacja Polska”. It mainly concerned paper chromatography and its capabilities, i.e., simplicity and high sensitivity. The possibility of separation and determination of analytes from the group of compounds posing numerous analytical problems was highlighted. This concerned, among others, amino acids and sugars, and biological samples such as human body fluids (22).

Another experimental study employing paper chromatography was published in „Acta Poloniae Pharmaceutica” in 1955. The report contained the results of chromatographic separation of alkaloids (23). On the other hand, the Polish journal „Farmacja Polska” published for the first time a chromatography-based article titled „Sulfonamides, improved method of separating using paper chromatography” in 1954 (24).

In the second half of the 1950s, the articles on the use of experimentally determined paper chromatography systems for analysis of different groups of phytochemical substances were published. *Inter alia*, the qualitative assessment of the content of flavones in tinctures prepared from various species of the genus *Hypericum* (25), alkaloids in different parts of the genus *Vinca* (26), and triterpene acids in a variety of herbal raw materials (27-29) was performed. Methodological works have also been published, e.g., „Methodological studies on the quantification of amino acids separated by chromatography on paper” (30) and „Chromatography of barbiturates” (31).

In 1960, a specific type of paper chromatography was described under the name *elatography*. The aim of this technique was to obtain chromatographic separation of products due to chemical reaction on the paper. It was the opposite of classical paper chromatography, wherein the analytes were separated from the mixture. In the *elatography* technique, the components of the mixture tested, reacted with the reagents which were in a solvent forming the mobile phase. The analysis was characterized by the formation of reaction products and the direct separation of components in a sequential order (32).

In the 1960s, paper chromatography was used to analyze, among others, the samples of raw materials of plant origin and pharmaceutical preparations derived therefrom (33-42) as well as in toxicological studies (43). PC was employed to assess the composition of excipients in pharmaceutical preparations (44, 45) and to evaluate the quality of both the medicinal products and magistral preparations (46-50). PC was also applied to analyze the plant protection products (51).

A loss of interest in paper chromatography, which occurred in the 1970s, was associated with the rapid development of modern planar technique, namely, thin layer chromatography. In the following years, paper chromatography was used sporadically (52-54), most frequently as a two-dimensional paper chromatography (17, 18, 55-57).

**Thin layer chromatography (TLC)**

In 1962, a review article titled „Thin-layer chromatography in the pharmaceutical analysis” was published in which TLC technique, named an *open column*, had been described as a sign of progress in the development of chromatographic techniques. The article emphasized that TLC combines the advantages of both column chromatography and paper chromatography as well as eliminates the disadvantages of each of these techniques. Detailed instructions were given on how to prepare the plates, and the method for plate analysis was described. The application notes related to TLC from foreign journals were also listed (58).
In 1963, two experimental works on employing TLC to investigate phytochemicals were published in „Farmacja Polska” and „Acta Poloniae Pharmaceutica” (59, 60). In the second half of the 1960s, the results of the analysis of alkaloid raw materials were primarily reported (61-65). Later, the attempts were made to apply TLC to study the stability of medicaments and medicinal substances (66-71) as well as to research the medicinal chemistry topics (72, 73). The articles dedicated to methodology (74) and new technical solutions (75, 76) were also published. The results of analysis conducted by using paper and thin layer chromatography (77, 78) were reported, while a comparative analysis of these results served as a basis for specifying the advantages of TLC, especially in terms of the significant reduction of the time of analysis (79).

In the 1970s, several scientific studies employing TLC were published annually in the Polish pharmaceutical journals. The authors of these works were not only the employees of pharmaceutical departments at the Polish medical institutions of higher education, but also the employees of the Institute of Medicines in Warsaw and control laboratories of the Board of Pharmacies. The published papers included the following topics: new chromatographic systems designed to investigate the properly prepared samples (80); determination of active compounds in pharmaceutical formulations (81-88); additives in drugs (89); and drug decomposition products (90). A number of studies concerning phytochemical research, including the research of herbal raw materials (91) and the assessment of the identity and quality of herbal medicines (92, 93), were also published.

In the 1980s and 1990s, the declining interest in thin layer chromatography was observed. Then, the most commonly used TLC method, was two-dimensional thin layer chromatography (57, 94-96). It was also used spectrophotometric technique to determine compounds previously isolated from TLC plates on which the chromatographic separation was carried out (97, 98). At the end of the 1970s, pharmaceutical laboratories started to use densitometry as a method of detection in TLC (99). The possibility of simultaneous qualitative and quantitative analysis resulted in an increase in the number of publications employing TLC as a method of investigation (100-102), for example, a few papers per year were published in „Acta Poloniae Pharmaceutica”. The number of such papers was similar to the number of publications in which the authors had used newer chromatographic techniques based on the mechanism of column chromatography, e.g., gas chromatography.

Gas chromatography (GC)

In 1962, a review article titled „Gas chromatography and its application” was published in „Farmacja Polska”. Gas chromatography was described as one of the greatest achievements of analytical chemistry in the post-war era (103). However, the first publication employing gas chromatography in experimental studies, titled „Study of the physico-chemical properties of oil from the seeds of Xanthium orientale L.”, was only printed in „Farmacja Polska” in 1968. Scientists from the Medical University of Gdansk reported on the application of „Chromatoprep” chromatograph, which had been constructed at the Faculty of Chemistry, Gdansk University of Technology (104).

Since then, articles on the application of gas chromatography in pharmaceutical research have been published regularly. In the late 1960s and 1970s, the publications primarily focused on the analysis of essential oils (105, 106). The variability of qualitative composition of oils in dependency on the plant harvest time (107, 108) and the content of pesticide residues were investigated (109). Many studies described the possibility of using gas chromatography to assess the composition of psychotropic drugs (110), and to determine the degradation products of medicinal substances in selected dosage forms (111, 112).

Since the 1980s, gas chromatography has become a leading tool for analyzing psychotropic drugs and narcotics in human blood (113-115). The determination of contaminants in herbal raw materials and the dosage forms obtained from these materials was also conducted (116, 117), as well as phytochemical researches (118). In 1990s, a mass spectrometer has become the most widely used detector, which enabled the use of gas chromatography in medicinal chemistry (119, 120). At present, gas chromatography is still in use, which results in some articles being published annually in „Acta Poloniae Pharmaceutica”. However, in terms of the number of publications, GC is inferior to the youngest chromatographic technique used in pharmaceutical analysis, namely, high performance liquid chromatography.

High performance liquid chromatography (HPLC)

In 1979, a review article concerning high pressure (performance) liquid chromatography was published in „Farmacja Polska” under the title „Chemically bonded stationary phases, and their use in pharmaceutical analysis by high performance liquid chromatography” (121). Two years later, the first paper
on experimental research, titled „High performance liquid chromatography (HPLC) analysis of piracetam next to its degradation products” (122), was published.

Initially, there were relatively few publications employing high performance liquid chromatography, which dealt with either the degradation products and impurities in pharmaceutical formulations (123-127), or the samples of human bodily fluids and biologically important molecules (128-131). HPLC was a comparative method, both for research using spectroscopic methods (132), as well as for an older chromatographic techniques, especially TLC (133, 134). High performance liquid chromatography was also used for quantitative analysis, as a complement to qualitative tests performed by TLC (135).

In the early days of the HPLC technique, analysts personally constructed chromatographs. An example can be a device created in the Department of Inorganic Chemistry at the Medical University of Lublin (136).

The rapid development in the field of practical application of HPLC took place only in the first decade of the 21st century. Since then, several articles employing this technique have been published each year in „Acta Poloniae Pharmaceutica”. The advanced methods of sample preparation and a wide range of detectors used in HPLC-based procedures allowed for studying chemical compounds with various structures, including active substances in plant material, dosage forms, and biological material.

DISCUSSION AND CONCLUSIONS

Nowadays, chromatographic techniques are considered the most popular analytical methods in pharmaceutical analysis (1, 2, 137). High performance liquid chromatography has become, in a relatively short time, the most commonly used chromatographic technique in pharmaceutical research. The coupling of constantly perfected and developed detection techniques to HPLC creates analytical methods that enable the investigations of the largest possible number of chemical compounds (4, 5, 137, 138). The methods of sample preparation prior to analysis are a critical factor in the application of high performance liquid chromatography to pharmaceutical analysis. The sample preparation allows the purification of the collected material, the enrichment of analytes, and the analysis of samples with complex matrices in which the analytes are present at trace level concentrations. This applies particularly well to the samples of biological material collected from a patient (1, 138, 139). The testimony to the rapidly growing popularity of HPLC is the number of monographs suggesting the use of this technique that can be found in the contemporary Polish pharmacopoeia. For example, „Polish Pharmacopoeia VI” (2002) (140) recommends the application of high performance liquid chromatography in 124 monographs, while this method was generally not considered in the previous „Polish Pharmacopoeia V” (1990) (141).

Gas chromatography is also used in pharmaceutical analysis, however, due to its limitations, it has never become a dominant tool. Moreover, its application is limited to the selected groups of chemical compounds (138, 142). The planar methods, such as capillary analysis and paper chromatography, should be considered historical techniques (143). Despite the fact that „Polish Pharmacopoeia IV” from 1965 contained 12 monographs recommending these analytical methods (144), the contemporary pharmacopoeia does not contain any.

Thin layer chromatography is a unique planar technique. For the half of a century, it has maintained a strong position, especially in the analysis of raw materials and herbal medicines. Although TLC is in many ways inferior to high performance liquid chromatography, it still provides rapid and simple testing (1, 3, 145). The popularity of thin layer chromatography in pharmaceutical analysis is reflected by the increasing number of pharmacopoeial monographs, ranging from 184 in „Polish Pharmacopoeia V” (141) to 558 in „Polish Pharmacopoeia VI” (140).

The aforementioned interdependencies between different chromatographic techniques can be characterized by the number of papers published.

![Figure 1. The number of publications describing the possibilities of using chromatographic techniques (based on articles published in the years 1952-2014 in „Acta Poloniae Pharmaceutica”, according to the PubMed database). PC - paper chromatography, TLC - thin layer chromatography, GC - gas chromatography, HPLC - high performance liquid chromatography.](image-url)
in pharmaceuticals journals. The first two publications dealing with the use of column and paper chromatography in experimental studies were published in „Acta Poloniae Pharmaceutica” in 1952. By the end of 2014, 557 publications employing chromatographic techniques and published in „Acta Poloniae Pharmaceutica” were registered in the PubMed database (Fig. 1). In 225 of these publications, thin layer chromatography was used. The aforementioned studies were published over the period of 52 years. On the other hand, at the end of 2014, the number of works employing high performance liquid chromatography was 184. However, they were published over the period of 30 years.

Currently, high performance liquid chromatography is the most popular and fastest growing chromatographic technique in the field of pharmaceutical analysis. Thin layer chromatography is still popular, but HPLC has surpassed it in terms of the number of publications published per year. On the other hand, gas chromatography has a stable position in pharmaceutical analysis. However, the limitations of this technique result in the publication of only a few studies employing GC per year. In contrast, paper chromatography can only be considered a technique of the past. It should be emphasized, however, that this technique was used for the longest time as indicated by the relevant articles published in the journal „Acta Poloniae Pharmaceutica”.

REFERENCES


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The increasing drug consumption in Lithuania and all over the world makes us think about the negative consequences - the risk of toxicity. Fast and accurate identification of material that caused the poisoning reduces the probability in death cases and makes easier to determine the main cause of death. The results have shown that the most appropriate systems of solvents for qualitative analysis by TLC method of the mixture consisting of alprazolam, codeine and paracetamol are: system “D” (trichloromethane : acetone : conc. ammonia = 55 : 40 : 5 (v/v/v)) and system “F” (trichloromethane : diethyl ether : isobutanol : conc. ammonia = 50 : 30 : 15 : 5 (v/v/v/v)). For qualitative analysis of the mixture consisting of alprazolam, codeine and paracetamol by HPLC method the chromatographic column ACE C18 (25 cm × 4.6 mm × 5 µm), gradient elution mode (mixture of 3% acetic acid and methanol and the flow rate 1 mL/min have been used. The injection volume was 10 µL. Photodiode array detector (210 ñ 240 nm range) has been used. UV absorption spectra of materials measured using photodiode array detector have been identical to those presented in the scientific literature.

Keywords: alprazolam, codeine, paracetamol, TLC, HPLC

INVESTIGATION OF A MIXTURE CONTAINING ALPRAZOLAM, CODEINE AND PARACETAMOL USING THIN-LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHODS

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Abstract: The increasing drug consumption in Lithuania and all over the world makes us think about the negative consequences of this social phenomenon. The consumption of medicines is closely linked to the risk of poisoning. Drug poisonings in Lithuania composed 41.5% in 2009 and 60.98% in 2011 in total statistics at all. (1). It was stated that the group of medicinal products, which caused the frequent poisonings, includes psychotropic medicines, non-narcotic analgesics, non-steroidal anti-inflammatory drugs and opioid analgesics. According to the local public health office data of 2011, 24.1% of the patients in Lithuania were poisoned by centrally acting drugs (2).

Benzodiazepine derivatives are commonly used in the nervous system acting drugs subgroup. Comparing statistical data of 2009 and 2012, the consumption of alprazolam has grown up mostly (44.82%) among other benzodiazepines. Alprazolam caused poisonings in 2011 in USA accounted for 29% of total poisonings data (3). Paracetamol was the commonly used agent in analgesic and antipyretic drugs subgroup from 2009 to 2012 in Lithuania. In 2012, the preparations of pure paracetamol composed 3.029 EDD/1000 resid./day and 5.756 EDD/1000 resid./day, in combination with other active substances. Paracetamol was very often determined among the poisonings caused by non-narcotic analgesics: Australia - 12% (2005-2006), USA - 9% of poisonings and 70% of fatal poisonings and 1103 deaths determined in UK (2008-2012) (2-4). Paracetamol is probably the most widely used agent for the purposes of suicide in USA (3). Codeine is marketed in Lithuania only in combination with paracetamol (named „Ultracod”) or in combination with paracetamol and caffeine (named „Solpadeine”) (5). The consumption of codeine phosphate hemihydrate in combination with paracetamol in 2012 composed 0.125 EDD/1000 resid./day. Comparing of 2009 and 2012 data, the consumption growth rate was 30.2% (6). Codeine is one of the most widely used non-medical purpose analgesics in the United States (7).

Alprazolam, codeine and paracetamol are widely used in Lithuania and all around the world.
medicinal products which may cause fatal poisonings when used in inappropriate doses. Moreover, the practice shows the trend of administration of following products, and thus the possibility of severe poisonings.

In order to optimize the work of toxicological laboratory in the case of determination of potential causes of poisoning, it is appropriate to use analytical methods which could help to identify a number of materials per analysis. Therefore, alprazolam, codeine and paracetamol can be used separately or in combination. For example: in the case of cold and depression, alprazolam and codeine combination is possible; in the case of cough - paracetamol and codeine. Because paracetamol and paracetamol/codeine containing medicinal preparations in Lithuania are sold without a prescription, the possibility of overdosage of these substances increases.

The aim of the work was to optimize the thin-layer chromatography and high performance liquid chromatography methods suitable for alprazolam, codeine and paracetamol mixture separation and qualitative analysis.

EXPERIMENTAL

Materials and Methods

Thin-layer chromatography (TLC) method

Chromatographic plates coated with sorption mass of silica gel were used for analysis (plates dimensions 20 × 20 cm, silica gel 60 F$_{254}$, Merck, Germany). A glass chambers with ground glass lids were used for chromatography. The samples of analyzed substance were taken using glass capillaries with a volume of 10 µL. The eluent systems were prepared by mixing variable volumes of solvents: ethanol, trichloromethane, diethyl ether, conc. ammonia, acetone, isobutanol, isopropanol. Chromatographic plates (after drying) were visualized using Dragendorff reagent (modified by Munje) or UV light lamp (254 nm, 365 nm). Test solutions were prepared by dissolution of standards - alprazolam, codeine and paracetamol (Sigma-Aldrich, USA) - in methanol.

In that case the standard solutions of alprazolam (AE), paracetamol (PE) and codeine (KE) were prepared of concentration 0.1 mg/mL. The mixture of components (APKE) was prepared by mixing of an equal parts (1 mL) of each standard solution.

High performance liquid chromatography (HPLC) method

For optimal determination conditions chromatograph Waters 2695 with a photodiode array detector (Waters 996, 210-400 nm wavelength range) was used. Separation of samples was performed using ACE C18 chromatographical column (25 cm × 4.6 mm) which sorbent particle size is 5 µm. Methanol, purified water (prepared by “Millipore” (USA) water purification system), 3% acetic acid aqueous solution, phosphate buffer (pH = 7), 0.1 % trifluoroacetic acid and acetonitrile were used for eluent systems preparation. Test solutions were prepared by dissolution of standards - alprazolam, codeine and paracetamol (Sigma-Aldrich, USA) - in methanol.

In that case the standard solutions of alprazolam (AE), paracetamol (PE) and codeine (KE) were prepared of concentration 0.1 mg/mL. The mixture of components (APKE) was prepared by mixing of an equal parts (1 mL) of each standard solution.

RESULTS AND DISCUSSION

Optimization of TLC method

Selection of the visualization reagent

After the finishing of tests, in order to evaluate which visualizer is most suitable for all three substances identification, two methods of spots visualization have been checked: spraying with Dragendorff reagent (modified by Munje) and visualization using UV light (254 nm, 365 nm).

In both cases the spots of all substances were clearly visible on the chromatographic plate. Use of UV light in comparison with Dragendorff reagent is less expensive, easier to apply and leads to a lower operative personal contact with the active chemical reagents.

Selection of the eluent

Searching for the more acceptable eluent, the components of solvents and their ratio were changed in several solvent systems. Solvent system suitability was assessed according to standard alprazolam, codeine and paracetamol solutions drift spots on the chromatogram. By comparison of these values 6 solvent systems (in volumetric ratios) were selected as suitable for the identification of compounds in a mixture:

- system „A“ - trichloromethane : diethyl ether : isopropanol = 35 : 35 : 30;
- system „B“ - trichloromethane : diethyl ether : isobutanol = 35 : 35 : 30;
- system „C“ - trichloromethane : diethyl ether : conc. ammonia = 45 : 45 : 10;
Investigation of a mixture containing alprazolam, codeine, and... 615

- system „E” - ethanol : acetone : conc. ammonia = 75 : 20 : 5;

Analysis of the solution of a mixture has been repeated using the different solvent systems five times. The statistical data evaluation has been performed by calculation of the arithmetic average X of the values of obtained R_f values, standard deviation SD, relative error RE at a confidence level of 0.95, confidence interval CI, when the error probability p < 0.05. (Table 1).

Table 1. Statistic evaluation of R_f values of the test substances (alprazolam, codeine, paracetamol) using solvent systems „A”, „B”, „C”, „D”, „E” and „F” in volumetric ratios.

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>Test substance</th>
<th>R_f average</th>
<th>Standard deviation (SD)</th>
<th>Relative error (RE)</th>
<th>Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>System „A”</td>
<td>alprazolam</td>
<td>0.58</td>
<td>0.008</td>
<td>0.004</td>
<td>0.58-0.59</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isopropanol 35 : 35 : 30</td>
<td>codeine</td>
<td>0.17</td>
<td>0.005</td>
<td>0.002</td>
<td>0.16-0.17</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.59</td>
<td>0.002</td>
<td>0.002</td>
<td>0.58-0.58</td>
</tr>
<tr>
<td>System „B”</td>
<td>alprazolam</td>
<td>0.39</td>
<td>0.009</td>
<td>0.004</td>
<td>0.39-0.41</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isobutanol 35 : 35 : 30</td>
<td>codeine</td>
<td>0.10</td>
<td>0.011</td>
<td>0.005</td>
<td>0.09-0.11</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.63</td>
<td>0.008</td>
<td>0.003</td>
<td>0.63-0.64</td>
</tr>
<tr>
<td>System „C”</td>
<td>alprazolam</td>
<td>0.92</td>
<td>0.008</td>
<td>0.004</td>
<td>0.91-0.93</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : conc. ammonia 45 : 45 : 10</td>
<td>codeine</td>
<td>0.92</td>
<td>0.005</td>
<td>0.002</td>
<td>0.91-0.92</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.78</td>
<td>0.007</td>
<td>0.003</td>
<td>0.78-0.79</td>
</tr>
<tr>
<td>System „D”</td>
<td>alprazolam</td>
<td>0.82</td>
<td>0.006</td>
<td>0.003</td>
<td>0.81-0.82</td>
</tr>
<tr>
<td>trichloromethane : acetone : conc. ammonia 55 : 40 : 5</td>
<td>codeine</td>
<td>0.69</td>
<td>0.007</td>
<td>0.003</td>
<td>0.6-0.80</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.49</td>
<td>0.008</td>
<td>0.004</td>
<td>0.4-0.80</td>
</tr>
<tr>
<td>System „E”</td>
<td>alprazolam</td>
<td>0.85</td>
<td>0.009</td>
<td>0.004</td>
<td>0.85-0.86</td>
</tr>
<tr>
<td>ethanol : acetone : conc. ammonia 75 : 20 : 5</td>
<td>codeine</td>
<td>0.72</td>
<td>0.0010</td>
<td>0.004</td>
<td>0.71-0.73</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.84</td>
<td>0.006</td>
<td>0.003</td>
<td>0.83-0.84</td>
</tr>
<tr>
<td>System „F”</td>
<td>alprazolam</td>
<td>0.86</td>
<td>0.004</td>
<td>0.002</td>
<td>0.85-0.86</td>
</tr>
<tr>
<td>trichloromethane : diethyl ether : isobutanol : conc. ammonia 50 : 30 : 15 : 5</td>
<td>codeine</td>
<td>0.75</td>
<td>0.008</td>
<td>0.004</td>
<td>0.74-0.75</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>0.45</td>
<td>0.007</td>
<td>0.003</td>
<td>0.44-0.45</td>
</tr>
</tbody>
</table>

Figure 1. The average R_f values of testing substances (alprazolam, codeine, paracetamol) in different systems of solvents.
The repeatability error of used for duplicate tests solvent systems does not exceed the permissible limit of 0.05, so they all are suitable for alprazolam, codeine and paracetamol mixture qualitative analysis.

From Figure 1 data it follows that the average \( R_f \) values of compounds in different solvent systems are not equivalent.

Application of solvent systems \( \text{ÑAì} \) and \( \text{ÑBì} \) shows the availability of analysis at a relatively low codeine \( R_f \) values (\( R_f < 0.17 \) and \( < 0.1 \), respectively), and its separation from the other components of the mixture is difficult. These two systems do not contain concentrated ammonia, which is present in other systems (\( \text{ÑCì}-\text{ÑFî} \)) and is important for efficient elution of codeine. Application of system \( \text{ÑAì} \) for a mixture analysis lead to alprazolam and paracetamol very close \( R_s \) values (\( R_s = 0.14 \)), thus this solvent system is suitable for alprazolam or paracetamol separation from codeine (\( R_s \approx 0.85 \) and \( 6.0 \), respectively). \( \text{ÑBì} \) solvent system completely separates alprazolam and paracetamol (\( R_s = 3.4 \)).

Comparison of \( \text{ÑAì} \) and \( \text{ÑBì} \) solvent systems advantage shows that the elution of alprazolam is greater...
with isopropanol than isobutanol (difference in \( R_f \) values > 0.19). For paracetamol elution difference between „A“ and „B“ systems is negligible (difference in \( R_f \) values < 0.4).

Using for analysis „C“ solvent system, codeine and alprazolam \( R_f \) values are close to each other (\( R_f = 0 \)) and there is no possibility to separate these components of a mixture from each other. Concentrated ammonia presented in solvent system „C“ significantly increased codeine \( R_f \) value in comparison with „A“ and „B“ solvent systems. „C“ system eluent is suitable for codeine or alprazolam separation from paracetamol (\( R_f = 2.8 \)).

Using for separation a mixture of substances „E“ solvent system, close \( R_f \) values for paracetamol and alprazolam could be calculated (\( R_f = 0.2 \)), therefore this system is not appropriate for alprazolam separation from paracetamol. „E“ system can be used to separate alprazolam or paracetamol from codeine (\( R_f = 2.6 \) and 2.4, respectively).

The most suitable solvent systems for alprazolam, codeine and paracetamol mixture separation are systems „D“ and „F“ (see Figs. 2 and 3). Using „D“ system as an eluent the mixture components can be completely separated from each other: alprazolam – codeine (\( R_s = 3.25 \)), alprazolam – paracetamol (\( R_s = 8.3 \)) and codeine – paracetamol (\( R_s = 5.0 \)). Similar results were obtained using as an eluent „F“ solvent system: alprazolam – codeine (\( R_s = 2.8 \)), alprazolam – paracetamol (\( R_s = 10.25 \)) and codeine – paracetamol (\( R_s = 7.5 \)).

**Optimization of HPLC method**

For the mixture consisting of alprazolam, codeine and paracetamol analysis by high-performance liquid chromatography Waters 2695 chromato-
A graph equipped with the photodiode array detector (DAD) was used. Using this detector the mixture was analyzed according to retention time value and UV light absorption spectrum. The application of these two criteria for qualitative assessment results in high accuracy.

To optimize the HPLC method, which can be used for identification of alprazolam, codeine and paracetamol in the mixture, primarily, the assessment of the standard solutions in UV absorption spectra was performed. The obtained spectra for substances during analysis corresponded to the standard materials UV light absorption spectra provided in the literature database (8) - the following values of absorption maxima were determined: at 221 nm wavelength for alprazolam (see Fig. 4), at 285 nm wavelength for paracetamol (see Fig. 5) and at 245 nm wavelength for codeine (see Fig. 6).

### Chromatographic column selection

In order to obtain the most suitable chromatographic column for the separation of the mixture of substances (APKE) several types of columns were used, but the best separation conditions were determined using ACE C18 (25 cm × 4 mm × 5 μm) chromatographic column.

### Eluent selection

During the separation and identification analysis, the mixture of standard component solutions (APKE) was investigated using eluents of different composition. After the experiments using the condi-

---

**Table 2. Eluent composition (gradient mode).**

<table>
<thead>
<tr>
<th>Chromatographic time (min)</th>
<th>3% acetic acid solution (A) %</th>
<th>Methanol (B) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>00:00</td>
<td>70.0</td>
<td>30.0</td>
</tr>
<tr>
<td>28:00</td>
<td>20.0</td>
<td>80.0</td>
</tr>
<tr>
<td>29:00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>31:00</td>
<td>10.0</td>
<td>90.0</td>
</tr>
<tr>
<td>32:00</td>
<td>70.0</td>
<td>30.0</td>
</tr>
</tbody>
</table>

**Table 3. Statistic evaluation of retention time (R_t) values of the test substances (alprazolam, codeine, paracetamol).**

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Test substance</th>
<th>Retention time (R_t)</th>
<th>Standard deviation (SD)</th>
<th>Relative error (RE)</th>
<th>Confidence interval (CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% acetic acid – methanol</td>
<td>alprazolam</td>
<td>29.81</td>
<td>0.009</td>
<td>0.004</td>
<td>29.81–29.83</td>
</tr>
<tr>
<td></td>
<td>codeine</td>
<td>6.78</td>
<td>0.008</td>
<td>0.003</td>
<td>6.77–6.79</td>
</tr>
<tr>
<td></td>
<td>paracetamol</td>
<td>7.92</td>
<td>0.008</td>
<td>0.003</td>
<td>7.91–7.93</td>
</tr>
</tbody>
</table>
Investigation of a mixture containing alprazolam, codeine, and... 619

In the scientific literature, the obtained results have not reached the maximum separation of substances, thus the conditions have been modified in order to obtain optimal results. The solvent systems were assessed by: the values of retention time of analytes, the peak symmetry and the baseline stability.

The separation of APKE was performed using as eluent a solvent system composed of 3% acetic acid aqueous solution (A) and methanol (B). The quantitative composition of the eluent was changed using gradient mode (Table 2).

During the separation analysis, the mobile phase flow rate was: 0.5, 1.0 and 1.5 mL/min. The optimal separation of substances was achieved under conditions when the eluent flow rate was 1.0 mL/min. The total chromatographic analysis time was 32 min (see Table 2, Fig. 7).

Mixture of alprazolam, codeine and paracetamol standards was rechromatographed under the specified conditions five times. The obtained data were evaluated statistically by calculation of an arithmetic average X of the retention time (Rt) values, standard deviation SD, relative error RE at a confidence level of 0.95, confidence interval, when the error probability \( p = 0.05 \). (Table 3).

During the chromatographic separation process of APKE as first codeine is eluted from chromatographic column (retention time (Rt) average is 6.78 min). Paracetamol is eluted second after codeine (retention time (Rt) average is 7.92 min) and finally, the elution of alprazolam occurs (retention time (Rt) average is 29.81 min) (Table 3).

After performed repeatable separations, the obtained results do not exceed the error probability \( p < 0.05 \), so the method is suitable for the mixture analysis.

**Separation method validation**

The methods obtained for the mixture components separation and identification have been validated using specificity criteria according to ICH guidelines.

TLC: the mixture components have been identified according to standard solutions Rt values (Table 1, Fig. 2, Fig. 3).

HPLC: the mixture of components have been identified according to standard solutions retention time (Rt) values (Table 3, Fig. 7) and UV spectra, recorded by photodiode array detector (DAD) and found in appropriate literature sources. (see Figs. 4-6).

**CONCLUSION**

Optimized thin-layer and high performance liquid chromatography methods are suitable for alprazolam, codeine and paracetamol mixture separation and qualitative analysis.

**REFERENCES**


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A SIMPLE AND SENSITIVE STABILITY-INDICATING UHPLC-DAD METHOD FOR THE DETERMINATION OF CEFETAMET PIVOXIL HYDROCHLORIDE

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2 Department of Modified Antibiotics, Institute of Biotechnology and Antibiotics, Starościancka 5, 02-516 Warszawa, Poland

Abstract: A fast and sensitive UHPLC-DAD method was developed and subsequently validated for determination of cefetamet pivoxil hydrochloride in the presence of its degradation products. The chromatographic separation was carried out on a Waters Acquity BEH C18, (2.1 × 100 mm, 1.7 µm) column. The mobile phase was composed of 0.1% formic acid and acetonitrile (40 : 60, v/v) at the flow rate 0.7 mL/min. The detection wavelength was 265 nm and the temperature was 30°C. Cefetamet pivoxil hydrochloride was susceptible to degradation under the influence of sodium hydroxide, hydrochloric acid and in the conditions of increased temperature and relative humidity. However, it was stable after irradiation, in increased temperature in dry air and in the presence of oxidizing agent. The developed UHPLC-DAD method was linear over the concentration range of 10–240 µg/mL (r² = 0.9999; n = 12). The obtained RSD values were less than 2%, demonstrating that the described procedure is precise. The accuracy was also confirmed (mean recoveries were 97.79-102.08%). Under applied chromatographic conditions LOD and LOQ values were 2.08 mg/mL and 6.29 mg/mL, respectively. The proposed method was successfully applied in determination of cefetamet pivoxil hydrochloride in aqueous solutions as well as in the solid state.

Keywords: UHPLC-DAD, cefetamet pivoxil hydrochloride, stability

Cefetamet pivoxil hydrochloride ((6R,7R)-7-[(2-amino-4-thiazolyl)-(methoxyimino)acetyl]-amino)-3-methyl-8-oxo-5-thia-1-azabicyclo [4.2.0] oct-2-ene-2-carboxylic acid (2,2-dimethyl-1-oxo-propoxy) methyl ester hydrochloride (CPH) (Fig. 1), is an oral third-generation cephalosporin. The active form of CPH - cefetamet is formed after oral administration by hydrolysis carried out by nonspecific mucosal esterases (1). CPH represents excellent microbiological in vitro activity against wide range of Gram-positive as well as Gram-negative respiratory pathogens such as Streptococcus pneumoniae, Haemophilus influenzae, β-hemolytic streptococci, Neisseria gonorrhoeae, and Enterobacteriaceae (2). Thanks to its significant stability against β-lactamases, corresponding with the presence of α-methoxyimino group, CPH is administered in the treatment of otitis media, pneumonia and in pharyngotonsilitis (3). It is also applied in the pharmacotherapy of both upper and lower respiratory tract and severe urinary tract infections, in the group of children and elderly patients (1, 4).

The structure determining the bactericidal activity of cephalosporins, which is generally considered as their most unstable domain, is β-lactam moiety. Moreover, it is widely reported that degradation products of β-lactam antibiotics are mostly responsible for side effects of this group of drugs. It was proved that CPH, similarly to other cephalosporins, is susceptible to degradation in

Figure 1. Chemical structure of cefetamet pivoxil hydrochloride

* Corresponding author: e-mail: pgarbacki@ump.edu.pl; phone: +48 61 854-66-49
aqueous solutions (5) and in the solid state (6). Therefore, fast, sensitive and accurate analytical assays appropriate for determination of active pharmaceutical ingredient (API) in the presence of its related products are expected. Although, many chromatographic methods for the determination of CPH in the bulk substance (7), in pharmaceutical dosage forms (7, 8) and in biological samples (9, 10) have been reported, they are based on mobile phases (e.g., phosphate buffers) which do not allow the transfer to LC-MS/MS technique.

All drugs during stability studies should be determined using stability-indicating methods (SIAMs) recommended by International Conference of Harmonization (ICH) guidelines (Q1A–R2) (ICH Q2B, validation of analytical procedures, methodology) (11). The stress tests are carried out to evaluate the influence of degrading factors on the stability of API in the solid state (at increased temperature, humidity and after irradiation) and in solutions (the impact of increased temperature, oxidizing agent, pH and buffers).

The aim of this work was to develop fast, sensitive and accurate UHPLC-DAD method suitable for determination CPH in the presence of its degradation products.

EXPERIMENTAL

Materials

Cefetamet pivoxil hydrochloride was received from the Institute of Biotechnology and Antibiotics in Warsaw, Poland. All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). High-quality pure water was prepared using the Millipore purification system (Millipore, Molsheim, France, model Exil SA 67120).

Chromatographic conditions

Chromatographic separation was carried on a Thermo Scientific UHPLC-UltiMate 3000 system. As a stationary phase Waters Acquity BEH C18, (2.1 × 100 mm, 1.7 µm) column was used. The mobile phase composed of 60 volumes of 0.1% formic acid and 40 volumes of acetonitrile. The flow rate was 0.7 mL/min and the wavelength of DAD detector was set at 265 nm. Separation was carried out at 30°C. The injected volume was 5 µL. The components of mobile phase and sample solutions were filtered through 0.2 µm nylon membranes.

Preparation of stock solutions

Stock solutions were prepared by dissolving 5.0 mg of CPH powder in small amount of methanol and later diluted to desired volume (25.0 mL) in distilled water. The final concentration 0.2 mg/mL was achieved. Stock solutions were stored in darkness at 4°C and proved stable during the time of the study.

Validation methodology

The proposed UHPLC procedure was validated according to the International Conference on Harmonization Guidelines (ICH Q2B, validation of analytical procedures, methodology) (11). The method

![Figure 2. The UHPLC chromatogram of cefetamet pivoxil hydrochloride (A, t_R = 1.570 min) in the presence of degradation products (B, t_R from 0.373 to 0.553 min) after incubation in RH ~ 76%, at 343 K for 10 days](image-url)
A simple and sensitive stability-indicating UHPLC-DAD method for... was validated with respect to selectivity, linearity, precision, accuracy, LOD, LOQ and robustness.

**Selectivity studies**

The selectivity of proposed chromatographic method was evaluated for non-degraded and for degraded CPH samples in aqueous solutions (acidic, basic, oxidative and thermal hydrolysis) as well as in the solid state (degradation under the influence of increased humidity, temperature and radiation). Photodiode array detection was carried out to evidence the selectivity of the procedure and to evaluate the homogeneity of CPH peaks.

**Linearity**

The linearity was confirmed by preparing twelve standard solutions of CPH in the concentration range 10.0-240.0 µg/mL (5-120% of the targeted concentration of CPH samples during forced degradation studies). Each standard solution was injected in triplicate to evaluate the reproducibility of detector response at each concentration level.

**Precision**

The precision of proposed analytical procedure was confirmed in relation to repeatability (intra-day) and intermediate precision (inter-day). In order to evaluate repeatability of the method six samples were determined during the same day for three concentration levels of CPH (160, 200 and 240 µg/mL). Inter-day precision was investigated by comparing the results obtained on two different days.

**Accuracy**

The determination of the accuracy of the developed UHPLC-DAD method was carried out by recovering CPH from the placebo. The recovery test was performed for three concentration levels of CPH: 160, 200 and 240 µg/mL. Each of abovementioned solutions was injected six times.

**Limits of detection (LOD) and quantification (LOQ)**

The LOD and LOQ were calculated from the regression equation of the CPH: LOD = 3.3 (Sy/σ), LOQ = 10 (Sy/σ), where Sy is a standard error and σ is the slope of the corresponding calibration curve.

**Robustness**

The robustness of the method was determined after changing the following experimental conditions: the composition of the mobile phase (concentration of acetonitrile in the range 38–42%, concent-

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### Table 1. Results of forced degradation studies.

| Stress conditions and time | Degradation (% of remained CPH) | Peak purity |<sup>a</sup> |
|----------------------------|---------------------------------|-------------|
| Water/343 K/94 h           | 51.3                            | 99.41       |
| Acidic/0.1 mol/L HCl/343 K/24 h | 21.5                           | 99.78       |
| Basic/0.1 mol/L NaOH/ambient temp./1 min | 1.2                          | 98.53       |
| Thermal/343 K/RH ~ 76%/10 days | 8.5                            | 98.62       |
| Thermal/343 K/RH = 0%/14 days | 84.6                           | 98.60       |
| Oxidizing/0.3% H<sub>2</sub>O<sub>2</sub>/ambient temp./2.5 h | 91.2                          | 99.28       |
| Oxidizing/1% H<sub>2</sub>O<sub>2</sub>/ambient temp./2.5 h | 82.4                          | 99.65       |
| Oxidizing/2% H<sub>2</sub>O<sub>2</sub>/ambient temp./2.5 h | 78.5                          | 99.97       |
| Oxidizing/5% H<sub>2</sub>O<sub>2</sub>/ambient temp./2.5 h | 67.1                          | 99.96       |
| Radiolytic/25 kGy            | 98.5                           | 99.58       |
| Radiolytic/400 kGy           | 87.5                           | 99.45       |

<sup>a</sup> Peak purity values in the range of 98.5–100 indicate a homogeneous peak.

### Table 2. Regression equation, standard deviation value, LOD and LOQ values of cefetamet pivoxil hydrochloride (concentration range: 10–240 µg/mL (n = 12).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression equation</td>
<td>y = (17153.09 ± 98.18) x – (0.2497 ± 0.1630)</td>
</tr>
<tr>
<td>Standard deviation (R2)</td>
<td>0.9999</td>
</tr>
<tr>
<td>LOD (µg/mL)</td>
<td>2.08</td>
</tr>
<tr>
<td>LOQ (µg/mL)</td>
<td>6.29</td>
</tr>
</tbody>
</table>
of formic acid in the range 0.05-0.15%), the mobile phase flow rate (flow rate in the range 0.68-0.72 mL/min), wavelength of absorption (265 ± 5 nm), temperature (30 ± 2°C). For each parameter change its influence on the retention time ($t_R$), resolution ($R_S$), area (A) and asymmetry of the peak was evaluated.

**Procedure for forced degradation study**

**Degradation in aqueous solution**

The degradation studies of CPH in aqueous solutions were carried out in the following conditions: in water at 343 K, in the solution of hydrochloric acid (0.1 mol/L) at 343 K, in the solution of sodium hydroxide (0.1 mol/L) at ambient temperature. Solutions were obtained by dissolving 5.0 mg of CPH in small amount of methanol and later diluted with either distilled water, hydrochloric acid or sodium hydroxide to achieve the concentration 0.2 mg/mL. At specified times, samples of the reaction solutions (1.0 mL), except the sodium hydroxide, were instantly cooled with a mixture of ice and water.

**Thermal degradation**

In order to achieve the thermal degradation in the solid state 5.0 mg samples of CPH were weighted in 5.0 mL vials and placed in heat chamber at 343 K in desiccator containing saturated solution of inorganic salt – sodium chloride (~76 % RH) and in a sand bath (dry air conditions). At specified time intervals, determined by the rate of degradation, the vials were removed, cooled to room temperature and their contents were dissolved in small amount of methanol and later diluted with distilled water to obtain the concentration 0.2 mg/mL.

**Oxidative degradation**

To perform oxidative degradation, 5.0 mg samples of CPH were accurately weighted and dissolved in small amount of methanol and then diluted with either 0.3, 1, 2 or 3% of a H$_2$O$_2$ solution to achieve the concentration 0.2 mg/mL. The abovementioned solutions were kept at ambient temperature.

**Radiolytic degradation**

Five mg of samples of CPH were weighed in 5.0 mL vials and closed with a plastic stopper. The samples in the vials were exposed to irradiation in a linear electron accelerator LAE 13/9 (electron beam 9.96 MeV and current intensity 6.2 1 A) till they absorbed a dose of 25 and 400 kGy. The vials were removed and their contents were dissolved in small amount of methanol and later diluted with distilled water to obtain the concentration 0.2 mg/mL.

**RESULTS AND DISCUSSION**

It was observed that the most satisfactory chromatographic parameters for determination of CPH in the presence of its degradation products were achieved using C18 1.7 µm column as a stationary phase and a mixture of 0.1% formic acid with acetonitrile (40 : 60, v/v) at the flow rate 0.7 mL/min as a mobile phase. The retention time of CPH was 1.570 min (Fig. 2), which is greatly shorter than values presented in available HPLC procedures (6.26-9.93 min) (5, 7). The purity of the peak and the asymmetry were 99.8% and 1.34, respectively. The selectivity studies, performed for non-degraded and degraded samples, confirmed that proposed UHPLC method is suitable for determination of CPH in the presence of its degradation products. The peak of CPH demonstrated satisfactory symmetry and was clearly separated from the peaks originating from degradants (Fig. 2). It was also observed that peak purity values were more than 98.5% for CPH at 265 nm, which proves that the degradation products were not interfering with the main peak (Table 1).

The calibration plot of developed chromatographic procedure is linear in the concentration range 10-240 µg/mL. The calibration curve was described by the equation $y = ac + b$ given in Table 2. The $b$ value, calculated from equation $y = ac + b$, was significant because it was higher than the critical value $t_b = b/S_b$. The intra- and inter-day precision was determined at three levels of initial concentration of CPH during stability studies: 80% (c = 320 µg/mL), 100% (c = 400 µg/mL) and 120% (c = 480 µg/mL).

<table>
<thead>
<tr>
<th>Spiked concentration (µg/mL)</th>
<th>Intra-day precision RSD (%)</th>
<th>Inter-day precision RSD (%)</th>
<th>Recovery RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160</td>
<td>0.13</td>
<td>0.09</td>
<td>102.08</td>
</tr>
<tr>
<td>200</td>
<td>0.43</td>
<td>0.14</td>
<td>99.42</td>
</tr>
<tr>
<td>240</td>
<td>0.60</td>
<td>0.13</td>
<td>97.79</td>
</tr>
</tbody>
</table>
The percentage RSD values were between 0.14-0.43%, demonstrating that developed UHPLC method is precise. Recovery values obtained from the recovery test showed that applied chromatographic procedure is accurate and reproducible. LOD and LOQ values were 2.08 mg/mL and 6.29 mg/mL, respectively. All of abovementioned validation parameters are collected in Tables 2 and 3.

The robustness studies were carried out to evaluate the influence of slight alternations of the following parameters: concentration of components and flow ratio of the mobile phase, detection wavelength and temperature, on the chromatographic separation. The results are collected in Table 4. It was proved that the changes of abovementioned parameters, except the concentration of acetonitrile, did not significantly affect the retention time, resolution, area and asymmetry of the CPH peak. Modification of the content of organic modifier in the mobile phase resulted in the changes of peak retention times.

**Results of forced degradation experiments**

According to R. Sehrawat et al., 20-80% of the investigated substance should be degraded during stability studies to confirm that proposed method is suitable to indicate stability (12). The literature reports the significant susceptibility of compounds containing β-lactam moiety to degradation caused by physical and chemical factors (13-15). CPH, similarly to other cephalosporins, was found to be vulnerable to decomposition in the presence of acids, bases and in conditions of increased temperature and relative humidity. The main degradation products had retention times of about 0.373-0.553 min (Fig. 2). As it was expected, CPH rapidly hydrolyzed in the presence of 0.1 mol/L NaOH (about 1.2% of the initial amount of CPH remained after 1 min). CPH was also degraded in the solution of hydrochloric acid (0.1 mol/L) at 343 K. It was noticed that the degradation rate of CPH in the solid state was strongly determined by the relative humidity. The percentage of remained drug stored in dry air at 343 K for 14 days was about ten times higher than in the case of sample exposed to the increased humidity at the same temperature for 10 days. The similar retention times of degradation products observed on chromatograms obtained for samples in the solid state as well as in solutions allow to suggest that in both cases the hydrolysis of amide bond in β-lactam structure occurs. The drug was also found to be fairly resistant to oxidative stress conditions. It was also noticed that the stability of CPH depends on the concentration of hydrogen peroxide. Cefetamet pivoxil hydrochloride was not found to be susceptible to radiolytic degradation (about 1.5 to 12.5% of CPH was decomposed). Despite the significant loss of the content of CPH during forced degradation experiments, no additional peaks on chromatograms were observed what can be associated with the fact that no degradation products containing chromophore structure were formed. However, structures which are the result of intermolecular interactions (characteristic to all cepham derivatives) can be noticed. The results of forced degradation experiments on CPH under various stress conditions are summarized in Table 1.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$t_r$</th>
<th>RS$^*$</th>
<th>A</th>
<th>Peak asymmetry$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal</td>
<td>1.573</td>
<td>4.92</td>
<td>38.8514</td>
<td>1.34</td>
</tr>
<tr>
<td>ACN = 38%</td>
<td>1.887</td>
<td>4.91</td>
<td>39.4674</td>
<td>1.34</td>
</tr>
<tr>
<td>ACN = 42%</td>
<td>1.330</td>
<td>4.37</td>
<td>38.4332</td>
<td>1.32</td>
</tr>
<tr>
<td>Formic acid = 0.05%</td>
<td>1.653</td>
<td>5.11</td>
<td>37.7831</td>
<td>1.25</td>
</tr>
<tr>
<td>Formic acid = 0.15%</td>
<td>1.527</td>
<td>5.27</td>
<td>39.4010</td>
<td>1.31</td>
</tr>
<tr>
<td>$f$ = 0.68 mL/min</td>
<td>1.623</td>
<td>5.06</td>
<td>40.2544</td>
<td>1.30</td>
</tr>
<tr>
<td>$f$ = 0.72 mL/min</td>
<td>1.527</td>
<td>5.16</td>
<td>37.7802</td>
<td>1.30</td>
</tr>
<tr>
<td>$\lambda$ = 260 nm</td>
<td>1.570</td>
<td>5.21</td>
<td>40.1812</td>
<td>1.36</td>
</tr>
<tr>
<td>$\lambda$ = 270 nm</td>
<td>1.577</td>
<td>5.02</td>
<td>37.0306</td>
<td>1.35</td>
</tr>
<tr>
<td>$T$ = 28°C</td>
<td>1.600</td>
<td>5.11</td>
<td>39.0548</td>
<td>1.38</td>
</tr>
<tr>
<td>$T$ = 32°C</td>
<td>1.543</td>
<td>5.03</td>
<td>38.6818</td>
<td>1.32</td>
</tr>
</tbody>
</table>

$^*$ Peaks are separated to baseline if resolution is > 1.5; $^*$ peak asymmetry < 1.50 indicates symmetry of peak.
CONCLUSIONS

Developed UHPLC-DAD method is suitable for the determination of CPH in the presence of its degradation products. The proposed chromatographic procedure demonstrates satisfying validation parameters such as: specificity, linearity, precision, accuracy and robustness. Thanks to the short time of analysis, the elimination of compounds containing ion pairs from the mobile phase and possibility to transfer to LC-MS technique the method meets the criteria of modern analytical approaches and can be used for routine quality control and stability indicating studies on CPH.

Declaration of interest

The authors declare that there are no conflicts of interest.

REFERENCES


Received: 24. 11. 2014
Trifluoperazine 2HCl (TFH), 10-[3-(4-methyl-1-piperazinyl) propyl]-2-(trifluoromethyl)-10H-phenothiazine dihydrochloride (Fig. 1), is a prominent compound in a large group of phenothiazine derivatives. Trifluoperazine (TFP) has central anti-adrenergic, antidopaminergic, and minimal anti-cholinergic effects. It is believed to work by blocking the postsynaptic D2 receptors in the mesocortical and mesolimbic pathways, and thus relieving or minimizing such symptoms of schizophrenia as hallucinations, delusions, and disorganized thought and speech (1).

TFH has official monographs in United States Pharmacopeia (2) and British Pharmacopeia (3). USP describes non aqueous potentiometric titration with perchloric acid, whereas BP also describes potentiometric method in which unreacted hydrochloric acid after treating with drug was titrated against NaOH in water : acetone medium. The reaction stoichiometry in both methods was evaluated by Job’s method of continuous variations and was found to be 1 : 2 (TFP : PA, TFP : DNP). The developed methods were successfully applied to the determination of TFP in pure form and commercial tablets with good accuracy and precision. Statistical comparison of the results was performed using Student’s t-test and F-ratio at 95% confidence level and the results showed no significant difference between the reference and proposed methods with regard to accuracy and precision. Further, the accuracy and reliability of the methods were confirmed by recovery studies via standard addition technique.

Keywords: trifluoperazine dihydrochloride, spectrophotometric assay, charge transfer complexation, pharmaceuticals

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**Figure 1. Structure of trifluoperazine dihydrochloride**

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spectrophotometry is the technique of choice even today because of its inherent simplicity, sensitivity, fair selectivity, accuracy and precision as well as cost-effectiveness. Visible spectrophotometric methods based on diverse reaction chemistries have earlier been proposed for the assay of TFP in pharmaceuticals. Revanasiddappa et al. (25) described a method based on the oxidation of TFH by a known excess of chloramine-T in a hydrochloric acid medium and determination of residual oxidant by its reaction with excess iodide resulting in liberation of iodine which was then made to react with starch followed by the measurement of the blue colored starch-iodine complex at 590 nm. Tehseen et al. (26) reported a method based on the reaction between drug and potassium persulfate, which gives an orange red colored complex having maximum absorbance at 480 nm. The orange red colored product formed by the reaction between drug and ammonium vanadate, measured at 525 nm was reported by Phillip and Satyanarayana (27). Kamalapurkar and Priolkar (28) reported a method based on the reaction between drug and Dragendorff’s reagent in acetone and the formed product was measured at 475 nm. A method based on the reaction of free base of drug with chloranil in dioxane-ethanol medium to give a violet colored compound which shows maximum absorption at 550 nm was reported by Gowda (29).

Most of the above visible spectrophotometric methods suffer from one or other disadvantage such as poor sensitivity (27), narrow linear range (25-27) and multi step reaction (25), as indicated in Table 1. Even though C-T reaction using chloranil (29) was reported, it requires mixture of solvents as working medium.

Nitrophenol derivatives such as 2,4-dinitrophenol (DNP) or picric acid (PA) are excellent π-acceptors (30, 31) and have been widely used in the assay of several drugs. This paper, for the first time, describes the application of these two reagents for the rapid, selective and sensitive spectrophotometric assay of TFP in bulk drug and in its dosage form. The methods involve the charge-transfer (C-T) complex formation reaction of the drug with PA (PA method) and DNP (DNP method) in dichloromethane to form intensely colored radical anions measurable at 410 nm in PA method and 415 nm in DNP method. The proposed methods are determined to be simple and rapid employing low cost reagents and instrument.

<table>
<thead>
<tr>
<th>No.</th>
<th>Reagent(s) used</th>
<th>Methodology</th>
<th>λ\text{max} (nm)</th>
<th>Linear range, (\mu\text{g/mL}) and ((\epsilon, \text{L mol}^{-1} \text{cm}^{-1}))</th>
<th>Remarks</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chloramine-T Starch-iodine complex measured</td>
<td>590</td>
<td>45-95 ng/mL, ((\epsilon = 4.07 \times 10^4))</td>
<td>Narrow linear range, multi-step reaction, require regular standardization</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Potassium persulfate Orange-red colored complex formed was measured</td>
<td>480</td>
<td>0.3-3.0 (NA)</td>
<td>Narrow linear range</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Ammonium vanadate The orange red colored product formed was measured</td>
<td>525</td>
<td>96.2-99.7 (NA)</td>
<td>Narrow linear range</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dragendorff’s reagent Formed chromogen was measured</td>
<td>475</td>
<td>10-100 (NA)</td>
<td>Use of expensive reagent</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Chloranil Charge-transfer complex was measured</td>
<td>550</td>
<td>–</td>
<td>–</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>a) PA b) DNP Formed radical anion was measured</td>
<td>410 415</td>
<td>1.5-24.0 ((1.03 \times 10^4)) 5.0-80.0 ((6.91 \times 10^4))</td>
<td>Simple, rapid, selective and use a single reagent. Single step reaction and no heating step involved.</td>
<td>Proposed methods</td>
<td></td>
</tr>
</tbody>
</table>

NA: not available.
EXPERIMENTAL

Apparatus

All absorbance measurements were made using a Systronics model 106 digital spectrophotometer (Systronics Ltd., Ahmedabad, India) with 1 cm path length matched quartz cells.

Chemicals and reagents

Pharmaceutical grade trifluoperazine dihydrochloride (TFH) was received from Sun Pharm. Indus. Ltd., Mumbai, India, as a gift and used as received. The following formulations were obtained from commercial sources and subjected to analysis: Psycalm-5 from Saga Lab., Ahmedabad, India, and Trazine-5 from Sun Pharm. Indus. Ltd., Mumbai, India. Dichloromethane (spectroscopic grade) was purchased from Merck, Mumbai, India.

(1) Picric acid (0.1%): Prepared by dissolving 0.1 g of picric acid (S.D. Fine Chem. Ltd., Mumbai, India) in 100 mL of dichloromethane and used for the assay in PA method.

(2) Dinitrophenol (0.05%): Prepared by dissolving 0.05 g of dinitrophenol (S.D. Fine Chem. Ltd., Mumbai, India) in 100 mL of dichloromethane and used for the assay in DNP method.

(3) Sodium hydroxide (1.0 M): Accurately weighed 1 g of pure NaOH (Merck, India) was dissolved in water; the solution was made up to 100 mL with water.

(4) Standard drug solution (TFP): Pure trifluoperazine dihydrochloride (23.6 mg) was dissolved in 20 mL water in a 125 mL separating funnel, 10 mL of 10% NaOH was added followed by 20 mL of dichloromethane. The content was shaken for 15 min. The lower organic layer was collected in a beaker containing anhydrous sodium sulfate. The water-free organic layer was transferred into a 100 mL calibrated flask and diluted to the volume with the same solvent to get 200 µg/mL with respect to TFP (hydrochloride free trifluoperazine). This solution was diluted appropriately with dichloromethane to get working concentrations of 30 µg/mL in PA method, and 100 µg/mL for use in DNP method.

Construction of calibration curves

PA method

Aliquots (0.25, 0.5, ……4.0 mL) of a standard TFP (30 µg/mL) solution were accurately transferred into a series of 5 mL calibration flasks. To each flask, 1 mL of 0.1% PA solution was added and the solution was made up to volume with dichloromethane. The content was mixed well and the absorbance was measured at 410 nm against a reagent blank.

DNP method

Different aliquots (0.25, 0.5………4.0 mL) of standard TFP solution (100 µg/mL) were accurately transferred into a series of 5 mL calibration flasks using a micro burette. One mL of 0.05% DNP solution was added to each flask and diluted to volume with dichloromethane. The content was mixed well and the absorbance was measured at 415 nm against a reagent blank.

Standard graph was prepared by plotting the absorbance versus drug concentration, and the concentration of the unknown was read from the calibration graph or computed from the respective regression equation.

Procedure for tablets

Twenty tablets were weighed and pulverized. The amount of tablet powder equivalent to 59.0 mg of TFH was transferred into a 100 mL volumetric flask. The content was shaken well with about 60 mL of water for 20 min and diluted to the mark with water. It was filtered using Whatman No. 42 filter.
paper. First 10 mL portion of the filtrate was discarded. Forty mL of the tablet extract (500 µg/mL) was quantitatively transferred to a separating funnel, pH was raised by adding 20 mL of 10% NaOH and the content was mixed well. The trifluoperazine base was extracted with three 20 mL portions of dichloromethane, the extract was passed over anhydrous sodium sulfate and collected in 100 mL volumetric flask, the volume was made up to mark with dichloromethane and the resulting solution (200 µg/mL TFP) was diluted to 30 and 100 µg/mL TFP for PA method and DNP method, respectively, and used for the assay.

Procedure for the analysis of placebo blank and synthetic mixture

A placebo blank containing starch (40 mg), acacia (35 mg), sodium citrate (35 mg), hydroxyl cellulose (35 mg), magnesium stearate (35 mg), talc (40 mg) and sodium alginate (35 mg) was prepared by mixing all the components into a homogeneous mixture. A 10 mg of the placebo blank was accurately weighed and its solution was prepared as described under ‘procedure for tablets’, and then subjected to analysis by following the general procedures. To the placebo blank of the composition described above, 11.8 mg of TFH was added and homogenized, transferred to a 100 mL calibrated flask and the solution was prepared as described under “procedure for tablets”, and then subjected to analysis by the procedures described above.

To the 10 mg of the placebo blank of the composition described above, 23.6 mg of TFH was added and homogenized, transferred to a 100 mL calibrated flask and the solution was prepared as described under “procedure for tablets”, and then subjected to analysis by the procedures described above.

RESULTS AND DISCUSSION

Absorption spectra

Trifluoperazine base (TFP) as n-electron donor with the π-acceptors such as PA and DNP results in the formation of C-T complexes and intense yellow colored radical anions which exhibit absorption maximum (Fig. 2) at 410 nm and 415 nm, respectively.

Reaction scheme

π-Acceptors such as trinitrophenol (picric acid) and dinitrophenol react with n-electron donor molecule to form charge transfer complexes which form radical ions (32-36). This reaction was used for the determination of some amine derivatives. Interest-

![Tentative reaction scheme for formation of radical anions](image-url)
ingly, application of picric acid for quantitative estimation of orphendrine citrate and phenolamine mesylate injections is official in the USP (37).

During the reaction, charge transfer complex of TFP and radical anions of PA and DNP are formed in respective methods. The absorption wavelength of radical anionic form of PA and DNP experiences bathochromic shift and yields yellow coloration in the visible region.

Because TFP has two tertiary amino groups in their molecular structure with the availability of non-bonding electron donors, it reacts with two moles of dinitrophenol or picric acid in dichloromethane to form a yellow colored radical anion. The interaction between TFP (D), an n-donor and nitrophenols (A), π-acceptors, is a charge transfer complexation reaction followed by the formation of radical ions (38) according to the following scheme.

\[ \text{D}^- + \text{A} \rightarrow [\text{D}^- \cdots \text{A}] \rightarrow \text{D}^{+} + \text{A}^- \]

[Donor + Acceptor → Complex → Radical ions]

The possible reaction pathway is shown in Figure 3.
Optimization of experimental variables

Many experimental variables which found to affect the color intensity and stability of the resulting complexes were optimized to achieve maximum sensitivity and adherence to Beer’s law.

Effect of reagent concentration

The optimum concentration of the reagent required to achieve maximum sensitivity of the developed color species in each method was ascertained by adding different amounts of the reagent PA or DNP to a fixed concentration of TFP. The results showed that 1.0 mL each of 0.1% PA and 0.05% DNP solution was optimum for the production of maximum and reproducible color intensity (Fig. 4).

Effect of solvent

In order to select a suitable solvent for preparation of the reagent solutions used in the study, the reagents were prepared separately in different solvents such as 1,4-dioxane, chloroform, acetonitrile, acetone, t-butanol, 2-propanol and dichloromethane. The reaction of TFP with PA or DNP was followed; dichloromethane was best suited for preparation of PA and DNP solutions. Similarly, the effect of the diluting solvent was studied for all methods and the results showed that the ideal diluting solvent to achieve maximum sensitivity and stability of the colored species was dichloromethane in both the methods (Fig. 5).

Effect of reaction time and stability of the C-T complexes

The optimum reaction times were determined by measuring the absorbance of the complex formed upon the addition of reagent solution to TFP solution at room temperature. The reaction of TFP with PA and DNP was instantaneous. The absorbance of the resulting C-T complexes remained stable for at least more than 40 h for PA and DNP methods.

Composition of the C-T complexes

The composition of the C-T complex was established by Job’s method of continuous variations (33) using equimolar concentrations of the drug (base form) and reagents (4.61 × 10^{-4} M in PA method and 2.09 × 10^{-4} M in DNP method). The results indicated that 1 : 2 (drug : reagent) complex is formed in both methods. Five solutions containing TFP and the reagent (PA or DNP) in various molar ratios, with a total volume of 5 mL in both methods were prepared. The absorbance of solutions was subsequently measured at 410 nm in PA method and at 415 nm in DNP method. The graphs of the results obtained (Fig. 6) gave a maximum at a molar ratio of \( X_{\text{max}} = 0.66 \) in both the methods, which indicated the formation of a 1 : 2 C-T complex between TFP and reagent (PA or DNP).

Method validation

The proposed methods were validated for linearity, sensitivity, selectivity, accuracy, precision, robustness, ruggedness and recovery according to the current ICH guidelines (39).

Linearity and sensitivity

Under the optimum conditions a linear relation was obtained between absorbance and concentration of TFP in the ranges given in Table 2. The calibration graph in each instance is described by the equation:

\[ Y = a + b \times X \]

where \( Y = \) absorbance, \( a = \) intercept, \( b = \) slope and \( X = \) concentration in µg/mL. The correlation coefficient, intercept and slope for the calibration data are summarized in Table 2. Sensitivity parameters such as apparent molar absorptivity and Sandell sen-

![Figure 6. Job’s plots obtained for (a) 4.61 × 10^{-4} M TFP in PA method, (b) 2.09 × 10^{-4} M TFP in DNP method](image-url)
sitivity (40) values, the limits of detection (LOD) and quantification (LOQ) are calculated according to the current ICH guidelines and compiled in Table 2. LOD and LOQ were calculated according to the same guidelines using the following formulae:

\[
LOD = 3.3 \times \frac{\sigma}{S} \quad \text{and} \quad LOQ = 10 \times \frac{\sigma}{S}
\]

where \(\sigma\) is the standard deviation of six reagent blank determinations and \(S\) is the slope of the calibration curve.

**Accuracy and precision**

In order to determine the accuracy and precision of the proposed methods, pure drug (TFP) solution at three different concentration levels (within the working range) were prepared and analyzed in seven replicates during the same day (intra-day precision) and on five consecutive days (inter-day precision) and the results are presented in Table 3. The percentage relative error (RE \%) was = 2.33 which indicate that the accuracy of the methods is satisfactory. Percentage relative standard deviation (RSD \%) for intra-day was = 2.15 and for inter-day it was = 2.26 indicating repeatability and usefulness of the proposed methods in the routine analysis.

**Selectivity**

The selectivity of the proposed methods for the analysis of TFP was evaluated by placebo blank and synthetic mixture analyses. The recommended pro-

### Table 2. Regression and analytical parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PA Method</th>
<th>NP Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\lambda_{max}), nm</td>
<td>410</td>
<td>415</td>
</tr>
<tr>
<td>Beer’s law limits (µg mL(^{-1}))</td>
<td>1.5-24.0</td>
<td>5.0-80.0</td>
</tr>
<tr>
<td>Molar absorptivity (L mol(^{-1}) cm(^{-1}))</td>
<td>1.03×10(^4)</td>
<td>6.91×10(^3)</td>
</tr>
<tr>
<td>Sandell sensitivity* (µg cm(^{-2}))</td>
<td>0.0395</td>
<td>0.0589</td>
</tr>
<tr>
<td>Limit of detection (µg mL(^{-1}))</td>
<td>0.34</td>
<td>0.25</td>
</tr>
<tr>
<td>Limit of quantification (µg mL(^{-1}))</td>
<td>1.03</td>
<td>0.76</td>
</tr>
<tr>
<td>Regression equation, Y**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept, (a)</td>
<td>-0.0084</td>
<td>0.0358</td>
</tr>
<tr>
<td>Slope, (b)</td>
<td>0.0265</td>
<td>0.0150</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.9997</td>
<td>0.9996</td>
</tr>
<tr>
<td>Standard deviation of intercept (S(_a))</td>
<td>0.00449</td>
<td>0.01001</td>
</tr>
<tr>
<td>Standard deviation of slope (S(_b))</td>
<td>0.00033</td>
<td>0.00022</td>
</tr>
</tbody>
</table>

*Limit of determination as the weight in µg/mL of solution, which corresponds to an absorbance of A = 0.001 measured in a cuvette of cross-sectional area 1 cm\(^2\) and l = 1 cm. **, where Y is the absorbance, a is the intercept, b is the slope and X is the concentration in µg/mL.

### Table 3. Evaluation of intra-day and inter-day precision and accuracy.

<table>
<thead>
<tr>
<th>Method</th>
<th>TFP taken (µg/mL)</th>
<th>Intra-day (n = 7)</th>
<th>Inter-day (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFP found (µg/mL)</td>
<td>%RSD(^*)</td>
<td>%RE(^*)</td>
</tr>
<tr>
<td>PA method</td>
<td>6.00</td>
<td>6.11</td>
<td>1.56</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>11.87</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>18.38</td>
<td>0.97</td>
</tr>
<tr>
<td>DNP method</td>
<td>20.0</td>
<td>19.74</td>
<td>2.03</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>39.79</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>60.54</td>
<td>0.84</td>
</tr>
</tbody>
</table>

Mean value of five determinations; \(^*\) Relative standard deviation (\%); \(^*\) Relative error (\%).
c edures were applied to the analysis of placebo
blank and the resulting absorbance readings in both
the methods were the same as that of the reagent
blank, confirming no interference from the placebo.
The analysis of synthetic mixture solution prepared
as described earlier yielded percent recoveries of
102.6 ± 1.86, 97.88 ± 2.13 (n = 5) for PA method
and DNP method, respectively. The results of this
study showed that the inactive ingredients did not
interfere in the assay indicating the high selectivity
of the proposed methods and its utility for routine
determination in pure drug and in tablets form.

Table 4. Robustness and ruggedness.

<table>
<thead>
<tr>
<th>Method</th>
<th>Parameter altered</th>
<th>Method robustness</th>
<th>Method ruggedness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TFP taken µg/mL</td>
<td>Reagent volume, mL</td>
<td>RSD, % (n = 3)</td>
<td>Inter-analysts RSD, % (n = 4)</td>
</tr>
<tr>
<td>PA method</td>
<td>6.00</td>
<td>1.23</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>1.06</td>
<td>1.39</td>
</tr>
<tr>
<td></td>
<td>18.0</td>
<td>0.81</td>
<td>1.17</td>
</tr>
<tr>
<td>DNP method</td>
<td>20.0</td>
<td>0.95</td>
<td>1.53</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>1.18</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>60.0</td>
<td>1.35</td>
<td>1.76</td>
</tr>
</tbody>
</table>

In both methods, the volume of reagent was 0.8, 1.0 and 1.2 mL.

Table 5. Results of analysis of tablets by the proposed methods.

<table>
<thead>
<tr>
<th>Tablet brand name</th>
<th>Label claim mg/tablet</th>
<th>Found (Percent of label claim ± SD)a</th>
<th>Reference method</th>
<th>Proposed methods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PA Method</td>
<td>DNP Method</td>
</tr>
<tr>
<td>Psycalm-5</td>
<td>5</td>
<td>102.3 ± 1.61</td>
<td>101.1 ± 0.75</td>
<td>99.72 ± 1.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t = 1.51</td>
<td>t = 2.42</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 2.15</td>
<td>F = 1.18</td>
</tr>
<tr>
<td>Trazine-5</td>
<td>5</td>
<td>98.67 ± 1.07</td>
<td>99.34 ± 1.76</td>
<td>100.4 ± 1.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>t = 0.81</td>
<td>t = 1.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F = 1.74</td>
<td>F = 2.38</td>
</tr>
</tbody>
</table>

aMean value of five determinations. Tabulated t-value at the 95% confidence level is 2.78. Tabulated F-value at the 95% confidence level is 6.39.

Table 6. Results of recovery study by standard addition method.

<table>
<thead>
<tr>
<th>Tablets studied</th>
<th>PA Method</th>
<th>DNP Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFP in tablets µg/mL</td>
<td>Pure TFP added, µg/mL</td>
<td>Total found, µg/mL</td>
</tr>
<tr>
<td>Psycalm-5</td>
<td>6.07</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>6.07</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>6.07</td>
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<tr>
<td>Trazine-5</td>
<td>5.96</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>5.96</td>
<td>6.0</td>
</tr>
<tr>
<td></td>
<td>5.96</td>
<td>9.0</td>
</tr>
</tbody>
</table>

aMean value of three determinations
Robustness and ruggedness

To evaluate the robustness of the methods, the experimental variable volume of reagent in both the methods were altered incrementally and the effect of this change on the absorbance of the C-T complexes was studied. The results of this study are presented in Table 4 and indicated that the proposed methods are robust. Method ruggedness was evaluated by performing the analysis following the recommended procedures by three different analysts and on three different cuvettes by the same analyst. From the % RSD values presented in Table 4, one can conclude that the proposed methods are rugged.

Application to analysis of tablets containing TFP

The proposed methods were successfully applied to the determination of TFP in two brands of tablets and the results are compiled in Table 5. The results obtained were statistically compared with those obtained by the reference method (3), by applying the Student’s t-test for accuracy and F-test for precision at 95% confidence level. The reference method involves acid-base titration to the potentiometric end point in acetone : water system. As can be seen from Table 5, the calculated t- and F-values at 95% confidence level did not exceed the tabulated values for four degrees of freedom. This indicates that there are no significant differences between the proposed methods and the reference method with respect to accuracy and precision.

Recovery study

The accuracy and validity of the proposed methods were further ascertained by performing recovery studies. Pre-analyzed tablet powder was spiked with pure TFP at three concentration levels (50, 100 and 150 % of that in tablet powder) and the total was analyzed by the proposed methods. The results of this study are presented in Table 6 and indicate that the excipients present in the tablets did not interfere in the assay.

CONCLUSION

Two simple, sensitive, extraction-free, rapid and cost-effective spectrophotometric methods based on charge transfer complex formation reaction are described for the determination of TFH. The methods were developed and validated as per the current ICH guidelines. The proposed methods utilize a single step reaction and a single solvent. No substantial differences among the proposed methods arose from analysis of the experimental results. The methods are free from interferences from the common excipients and additives. The statistical parameters and the recovery data reveal good accuracy and precision of the methods. These methods can be used as general methods for the determination of TFP in bulk powder and tablets, and have many advantages over the separation techniques such as HPLC. Hence, the methods can be used in routine analysis of drug in quality control laboratories.

REFERENCES

2. The United States Pharmacopoeia XXIV Revision, the National Formulary XIX, USP Convention, Rockville 2007.

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Polish Pharmaceutical Society

Human herpesvirus type 1 (HHV-1) commonly known as Herpes simplex virus type 1 (HSV-1) belongs to the Alphaherpesvirinae subfamily of Herpesviridae family.

Acyclovir (ACV) is the first selective inhibitor of alphaherpesviruses. It was discovered by G. Elion and H. Scheaffer in 1974 and became the treatment of choice for HHV-1 and HHV-2 infections. Until now, ACV is the most widely prescribed antiviral drug in the world. The drug is similar in structure to a DNA component – guanine deoxyriboside and is activated by viral thymidine kinase (TK) to its active form.

TK is a 376-amino acid protein, encoded by UL23 gene. In infected host cells ACV is phosphorylated by thymidine kinase to acyclovir monophosphate, host cellular enzymes, further, phosphorylate this drug and convert it to its active triphosphate form. The final product leads to irreversible inhibition of viral DNA synthesis by chain termination of viral DNA strands. ACV is well-tolerated, side effects are not frequent. Resistance of HHV-1 to ACV is related to mutations within UL 23 (thymidine kinase; TK) and UL30 (DNA polymerase) genes. ACV-resistant HHV infections are rare in immunocompetent hosts (from 0.1 to 1.0%). In immunocompromised patients, the prevalence of strains with reduced susceptibility to ACV depends on the reason of immunosuppression and ranges from 3.5 to 30% (1-5). The first clinical cases of acyclovir-resistant herpes simplex viruses were described in 1982, shortly after the initial use of intravenously administered acyclovir. It is well known that prolonged use of ACV is the most important risk factor for the selection of resistant strains. Drug-resistant strains have also been isolated in the absence of a known history of acyclovir treatment (1).

The second-line antiviral agents for HHV-1 infections is foscarnet (FOS) and cidofovir (CDV). Foscarnet is an organic analogue of inorganic pyrophosphate; directly inhibits DNA polymerase by blocking the pyrophosphate binding site and preventing cleavage of pyrophosphate from deoxynucleotide triphosphates.

Oral bioavailability is low, so foscarnet must be administered intravenously. The most common adverse effects of FOS are: seizures, fever, nausea, diarrhea, headache, leucopenia, nephrotoxicity and metabolic derangements.

IN VITRO INHIBITION OF HHV-1 REPLICATION BY INOSINE PRANOBEX AND INTERFERON-α

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Abstract: Key issues in the development of novel antivirals are the emergence of resistant strains. The development of new drugs effective against herpes diseases has proven to be both difficult and time-consuming. Some alternative may be to optimize the efficacy and selectivity of existing antiviral drugs or combining them with other well known agents. Inosine pranobex exerts a direct antiviral effect as well as secondary effect to its immunomodulatory activity. We found that increasing concentrations of inosine pranobex (50-400 µg/mL) produced progressively growing inhibitory effect on HHV-1 replication, following infection of different cell lines. The combination of 1000 IU/mL IFN-α and inosine pranobex also resulted in enhanced anti-HHV activity. Immunotherapy may be beneficial for patients from whom strains resistant to currently known antiviral drugs have been isolated.

Keywords: acyclovir, human herpesvirus type 1, inosine pranobex, interferon-α

637

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Resistance to FOS can be developed quickly after a relatively short period of use. Most FOS resistant clinical HSV isolates contain single base substitutions in conserved regions II, III, VI, or VII and in a non-conserved region of the DNA pol gene.

Cidofovir is an acyclic phosphonate nucleotide analogue that has demonstrated potent, broad-spectrum activity against double-stranded DNA viruses. Only 2–26% of CDV is absorbed after oral administration, so cidofovir should be administered intravenously in the clinical management of patients. Because the most important adverse reaction during the therapy of cidofovir is nephrotoxicity, CDV is contraindicated in patients with moderate to severe preexisting renal dysfunction. Resistance is now rare, mostly due to mutations within the viral DNA pol gene (1, 4, 6, 7).

Common occurrences of serious HHV infections in both immunocompetent and immunocompromised patients, a small number of available antiviral drugs and the emergence of drug resistance necessitate research for new antiviral agents that work through different mechanisms of action or biologically active products which can stimulate immune responses against herpesviral infection. Another possible option is to search a new applications of available drugs and co-administration them to elicit additive or even synergistic effect (1, 7-11).

The immune system can be manipulated non-specifically by immunomodulation. There are many well-known substances capable of enhancing host defence mechanisms to provide protection against infection. One of them are inosine pranobex (isoprinosine) and interferons (12).

Inosine pranobex exerts an antiviral effects which are both direct and secondary to immunomodulatory activity. The mechanism of action in human body is still unclear but numerous studies have demonstrated that this drug exhibit pleiotropic effect. Inosine pranobex can augment the production of cytokines such as interleukin-1 (IL-1) and interleukin-2 (IL-2). It increases the production of interleukin 12 (IL-12), INF-γ and decreases interleukin-3 (IL-3) and interleukin-4 (IL-4) production in vitro. Inosine pranobex normalizes the cell-mediated immunity stimulating T-cell differentiation into cytotoxic T cells and T-helper cells and increasing cytokine production. It also increases the humoral immune response by stimulating the differentiation of B-lymphocytes into plasma cells and by enhancing antibody production. The agent potentiates neutrophil, monocyte, macrophage chemotaxis and phagocytosis. Inosine pranobex augments NK activity and inhibits viral replication as well. In in vivo studies inosine pranobex reduced severity of symptoms and shortened the duration of some viral infections. It has been found to be useful in treatment of several viral diseases such as herpes simplex, rhinovirus infections and herpes zoster, influenza, genital warts, EBV infection (12-18).

Combination of inosine pranobex and IFN-α appear to be effective treatment of the persistent meases complication of the central nervous system – subacute sclerosing panencephalitis (SSPE) (13, 19, 20). It has been reported that inosine pranobex possesses a weak anti-HIV-1 activity in human peripheral blood mononuclear cells (PBMC) and H9 cells and restores in vitro the impaired T-helper cells from patients with AIDS-related complex (21). Inosine pranobex is virtually non-toxic. After prolonged treatment with high doses nausea is occasionally observed (13).

Interferons are antiviral proteins (cytokines) that can be formulated from purified, natural, human interferons or produced using recombinant DNA techniques. They constitute a family of cytokines capable of inducing an antiviral state within a cells. They are potent and pleiotropic immune modulators and can inhibit replication of many viruses. The mechanism of action of interferons is complex – they can interfere with almost any stage of viral attack.

About 55 years ago, the first antiviral drug ido-deoxyuridine (IDU) was described. This compound becomes the first antiviral drug to be licensed for use in the treatment of HHV infections. Now, there are more than 50 licensed antiviral compounds, but half of them are used for the treatment of HIV infection. The discovery of effective antiviral agents has been facilitated by advances in the fields of chemistry, molecular biology and virology. Key issues in the development of novel antivirals are the emergence of resistant strains. The development of new drugs effective against human viral diseases has proven to be both difficult and time-consuming. Some alternative may be to optimize the efficacy and selectivity of existing antiviral drugs or combining them with other well known agents (7, 9, 12).

The aim of this study was to evaluate in vitro inhibition of three strains of HHV-1 replication by inosine pranobex and interferon-α. In particular, the study was focused on the problem if compounds may act specifically against different strains of human herpesviruses type 1.

EXPERIMENTAL

Compounds

Inosine pranobex (isoprinosine) was kindly provided by Gedeon Richter (Poland). Inosine pra-
In vitro inhibition of HHV-1 replication by inosine... 639

nobex (IP) is a synthetic compound formed from the p-acetamido benzoate salt of N-N dimethylamino-2-propanol, and inosine in a 3:1 molar ratio. IP is highly soluble in water and chemically stable. The concentrations used in tissue culture studies were non-toxic and ranged from 50 to 400 µg/mL. Concentrations of IP were chosen according to the toxicity examination. Interferon used in the studies was IFN-α-2a (Roche) at final concentrations 100 and 1000 IU/mL. Doses of IFN-α were chosen based on a search of the literature (22).

Viruses

The viral strains used in this study were: standard (laboratory, reference) strain of human herpesvirus type 1 McIntyre (HHV-1MC) – sensitive to acyclovir and two resistant to acyclovir clinical isolates (HHV-1H3a, HHV-1f12k). HHV-1H3a was obtained from an orofacial lesions from a patient with hematologic disorders. HHV-1f12k was obtained from - previously treated with aciclovir - woman with recurrent genital herpes.

Susceptibility to antiviral drugs (ACV and CDV) was assessed in vitro by phenotypic and genotypic assays. The yield reduction assay (YRA) which evaluates the ability of the compound to inhibit virus production in cell culture was applied in phenotypic tests. The Reed-Muench statistical method was used to determine the 50% end point (IC50).

HHV-1MC is sensitive to acyclovir, the IC50 value was 0.68 µg/mL. Clinical isolates are resistant to both acyclovir and cidofovir. The IC50 value of ACV for both clinical strains of HHV-1 were > 250 µg/mL, and IC50 of CDV for HHV-1H3a and HHV-1f12k were > 500 µg/mL. The thymidine kinase (TK) gene (UL23) sequence (352 bp) from HHV-1 clinical isolates was amplified and detected using the PCR. Using this genotypic methods we found that one of the phenotypically resistant strains (HHV-1H3a) shows changes in the gene encoding thymidine kinase, which is required for the phosphorylation of nucleoside analogs.

Tissue cultures

Antiviral activity of tested compounds have been compared in various cell lines. Viruses were propagated in human larynx carcinoma cells HEP-2 (ATCC, CCL-23) and primary human lung fibroblasts HEL 299 (ATCC, CCL-137). HEL 299 cells were cultured in Dulbecco’s modified Eagles medium (DMEM, Sigma-Aldrich) containing 10% fetal bovine serum (Gibco Life Technologies, UK) and 1% penicillin/streptomycin antibiotics (Gibco Life Technologies, UK). HEP-2 cells were grown and maintained in Eagle’s medium essential medium (Biomed, Poland). The cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

Cytotoxicity assay

This test was performed with HEP-2, HEL 299 and A549 (human lung adenocarcinoma epithelial cell line; ATCC, CCL185). The cytotoxicity of the inosine pranobex (at doses of 25-800 µg/mL) and IFN-α (at concentration of 100 and 1000 IU/mL) against three cell lines was assessed visually using light, inverted microscopy Olympus CK2 (Olympus Corp., Germany) and by the MTT colorimetric assay. The assay determines the ability of viable cells to convert a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) into an insoluble formazan precipitate. Cells were incubated in 96-microwell plates. After incubation of cells for 24 h, inosine pranobex and/or IFN-α were added (at doses given above) and cultured further for 24 and 48 h. In controls the cells were cultured without any of the tested drugs. All experiments were performed in triplicates. Absorbance values of examined samples was read spectrophotometrically at a wavelength of 490 nm on a reader (Reader 230, Organon Technica Turnhout, Belgium).

Antiviral assay

The antiviral effects of IFN-α and inosine pranobex were assessed in vitro by phenotypic assays. The antiviral activity of inosine pranobex was tested using series of non-toxic concentrations (50-400 µg/mL) diluted in an assay medium. IFN-α was used at final concentrations: 100 and 1000 IU/mL. To investigate the antiviral activity, cell cultures in microwell plates were infected with HHV-1 strains (0.01 TCID50/cell) for 60 min at 37°C. After the absorption, the inoculum was aspirated and fresh culture medium containing IP, IFN-α or both agents were added. Next, the cells were incubated for 48 h. The yield reduction assay (YRA) which evaluates the ability of the compounds to inhibit virus production in cell culture was applied. The cytopathic effect of the virus was evaluated by means of light, inverted microscopy, 48 h after infection of cultures with viruses. The Reed-Muench statistical method was used to determine the 50% end point (IC50), which was the lowest concentration of the tested drugs that reduce the viral infections of the control to a 50%.

Antiviral effect was estimated according to the reduction of the herpesvirus titer in the presence of
compounds with the controlled one. TCID_{50}/mL was calculated, it is a median tissue culture infective dose; that amount of a pathogenic agent that will produce pathological change in 50% of cell cultures inoculated. Each analysis was performed at least in triplicate. The results were statistically evaluated using Pearson’s correlation method to measure the correlation between the doses of IP and the titers of viruses. Calculated titers of viruses were evaluated by using a two-tailed Student’s t-test to compare two small sets of quantitative data (TCID_{50}, the measure of infectious virus titers).

The Wilcoxon signed rank sum test was also used to analyze differences between the INF/IP treated infected cells and the control group (infected cells treated only IP) in terms of efficacy to reduction of viral titer. A value of p < 0.05 was considered as statistically significant.

RESULTS

Cytotoxity assay

In this study, the effect of IP and IFN-α was assessed in in vitro experiments. Three different cell lines were exposed to varying concentrations of each compound (IP at doses of 25-800 µg/mL and IFN-α at concentration of 100 and 1000 IU/mL) for 48 h. The cytotoxicity was measured microscopically and by the MTT assay. There were no morphological changes in cell cultures treated with IP and IFN-α in the examined concentrations. Visual assessment of the cell monolayer showed no toxic effect in the presence of IP and IFN-α. MTT cytotoxicity assay confirmed earlier microscopic observations. The viability of cells in the presence of tested compounds was higher than 95%.

The compounds at all tested concentrations were nontoxic to A549, HEp-2 and HEL 299 cell lines. For this reason the TC_{50} (the toxic drug concentration which caused the reduction of viable cell numbers by 50%) and SI (selectivity index; CC_{50} to IC_{50} value) were not calculated.

Antiviral assay

Increasing concentrations of inosine pranobex (50-400 µg/mL) produced progressively greater inhibition of HHV-1_{MC} and HHV-1_{H3a} replication following infection of HEp-2 and inhibition the growth of HHV-1_{MC} in HEL 299 cell cultures (Pearson’s correlation, p < 0.05).

A summary of the inhibitory effects of IP on virus replication in HEL 299 and HEp-2 cells is shown in Table 1. When IFN-α alone was added to HHV-1 infected cells, only a weak antiviral effect was detected even at concentration as high as 1000 IU/mL (Figs. 1, 2). INF-α in the concentration 1000 IU/mL caused only about 1 log reduction in titers of HHV compared to that of the controls. Results were similar in the case of reference and clinical HHV strains (Fig. 2).

The inosine pranobex, when used at a concentration of 400 µg/mL, markedly inhibited the replication of HHV-1_{MC} in HEL 299 cell line. The combined treatment with IP (400 µg/mL) and INF-α (1000 IU/mL) significantly inhibited multiplication of the HHV-1_{MC} in HEL 299 cell culture as compared to the control where the virus has not been exposed to IP and/or IFN-α (Student’s t-test, p < 0.05). The average viral titer was inhibited by about 3 logarithms in comparison to the control and below 1 logarithm in comparison to the viral titer after exposition to the IP (400 µg/mL) alone.

Otherwise, the reduction of the HHV-1_{MC}, HHV-1_{H3a} and HHV-1_{F12K} titers in HEp-2 cell line after applying IP 400 µg/mL and α-INF (1000 IU/mL), in comparison to the viral titer in the con-

<table>
<thead>
<tr>
<th>Cell line →</th>
<th>HEL299</th>
<th>HEp-2</th>
<th>HEp-2</th>
<th>HEp-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus →</td>
<td>HHV-1_{MC}</td>
<td>HHV-1_{MC}</td>
<td>HHV-1_{H3a}</td>
<td>HHV-1_{F12K}</td>
</tr>
<tr>
<td>TCID_{50}/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>7.50E+05</td>
<td>9.30E+05</td>
<td>9.30E+05</td>
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</tr>
<tr>
<td>IP 50 µg/mL</td>
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<td>9.30E+05</td>
<td>9.30E+05</td>
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</tr>
<tr>
<td>IP 100 µg/mL</td>
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<tr>
<td>Pearson’s r</td>
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<td>-0.92</td>
<td>-0.92</td>
<td>-0.77</td>
</tr>
<tr>
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<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p = 0.1</td>
</tr>
</tbody>
</table>
In vitro inhibition of HHV-1 replication by inosine...

Inosine pranobex (IP) 400 µg/mL and IFN-α (1000 IU/mL) reduced the HHV-1MC, HHV-1H3a, and HHV-1F12K titers in HEp-2 cells by more than 1.5 logarithms (Student’s t-test, p < 0.05). The reduction of HHV-1MC, HHV-1H3a, and HHV-1F12K titers in HEL-299 cultures after treatment with IP (400 µg/mL) and IFN-α (1000 IU/mL), compared to the viral titer after applying inosine pranobex 400 µg/mL, was reduced by below 1 logarithm (Fig. 2).

Combination of IP and IFN-α display higher efficacy than either treatment alone and may increase therapeutic effect without augmenting toxic effects. Results are statistically significant only for HHV-1MC treated simultaneously with maximal doses of IP and INF-α in HEL-299 culture (Wilcoxon signed-rank test, p < 0.05).

The antiviral activity of the tested compounds was also analyzed on the basis of IC50 values (Table 2). The IC50 values of IP against HHV-1MC standard strain were 513.56 µg/mL (HEL 299) and 886.86 µg/mL (HEp-2).
The IC₅₀ value of IP alone against the clinical strain HHV-1McIntyre was 883.98 µg/mL (HEp-2). In contrast, dose-related response for clinical isolate HHV-1H3a in HEp-2 cell line was weaker. The IC₅₀ value as high as 1047.65 µg/mL was observed.

When infected cells were treated with IFN-α and IP simultaneously, an enhanced antiviral activity was found. Application of IFN-α (1000 IU/mL) with IP after infection of HEL 299 and HEp-2 with HHV-1Mc reduced the IC₅₀ to 378.71 µg/mL and 445.51 µg/mL, respectively.

The combination of 1000 UI/mL IFN-α and IP also resulted in enhanced anti-HHV clinical strains activity but less efficiently. IC₅₀ were reduced to 923.02 µg/mL (HHV-1f12k) and 810.81 µg/mL (HHV-1H3a) (Table 2). The strongest inhibitory effect on viral replication was observed after combined of addition maximal doses of IP and IFN-α to the HHV-1Mc infected HEp-2 culture. IC₅₀ has been reduced by approximately 50%.

DISCUSSION AND CONCLUSIONS

Infectious diseases are known since ancient time. The development of antiviral agents has progressed slowly. Numerous compounds have been evaluated for antiviral activity in cell cultures and in animals studies, but only a small number of them have been approved for human use (23-25). There are some anti-herpes medications; however, there is no cure for herpes infection. Antiviral drugs can reduce the frequency, duration and severity of outbreaks, as well as asymptomatic shedding of virus (1). Idoxuridine (5-iodo-2-deoxyuridine) was the first medication to be approved (1962) against herpesviral infection. Because of systemic toxicity, its use was restricted to the topical use in the herpes keratitis (26). The another drug used in the treatment of primary and secondary keratoconjunctivitis caused by HHV-1 was trifluorothymidine (5-trifluoromethyl-2-deoxyuridine, TFT). The next achievement was the introduction of vidarabine for treatment of herpes encephalitis - a serious life-threatening disease. Now, because of the many side effects and adverse reactions it is used only in keratitis caused by HHV-1 which has not responded to topical idoxuridine or when toxic or hypersensitivity reactions due to idoxuridine have occurred (24, 26).

Acyclovir (ACV, 9-(2-hydroxyethoxy)methyl guanine) was the first selective inhibitor of HHV replication. Having one of the most remarkable safety profiles of any antiviral agents, acyclovir became the treatment of choice for all HHV-1 infections. Unfortunately, shortly after the initial use of intravenously administered acyclovir the first clinical cases of resistant strain were reported. Another antiherpespoviral drugs (cidofovir and foscarnet) have a low bioavailability so they are preferentially administered intravenously and cause a lot of serious adverse reactions during the therapy (1, 27). No vaccine is currently available to prevent herpes infections.

Consequently, there is a great need for the development of new, safe, orally bioavailable drugs. The design and synthesis of anti-herpesvirus agents that possess a mechanism of action different than currently used compounds seems to be especially desirable (9, 10, 28-30). It is well known that the course of viral infection, the severity of symptoms, response to treatment and the ability to select resistant mutants depends on the patient’s immunocompetence.

Table 2. The IC₅₀ values after application of inosine pranobex (IP) and inosine pranobex with interferon-α (IFN-α) in virus-infected cultures of HEL 299 and HEp-2 cells.

<table>
<thead>
<tr>
<th>Cell line →</th>
<th>HEL 299</th>
<th>HEP-2</th>
<th>HEP-2</th>
<th>HEP-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus →</td>
<td>HHV-1McIntyre</td>
<td>HHV-1McIntyre</td>
<td>HHV-1H3a</td>
<td>HHV-1F12k</td>
</tr>
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<td>IP (50-400 µg/mL)</td>
<td>IC₅₀ (513.56)</td>
<td>IC₅₀ (569.52-464.56)</td>
<td>IC₅₀ (886.86)</td>
<td>IC₅₀ (883.98)</td>
</tr>
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<td>513.56</td>
<td>569.52-464.56</td>
<td>886.86</td>
<td>883.98</td>
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<tr>
<td></td>
<td>513.56</td>
<td>569.52-464.56</td>
<td>886.86</td>
<td>883.98</td>
</tr>
<tr>
<td>IP (50-400 µg/mL)/IFN-α (100 IU/mL)</td>
<td>IC₅₀ (397.61)</td>
<td>IC₅₀ (561.60-289.87)</td>
<td>IC₅₀ (884.87)</td>
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<td></td>
<td>397.61</td>
<td>561.60-289.87</td>
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<td>561.60-289.87</td>
<td>884.87</td>
<td>883.54</td>
</tr>
<tr>
<td>IP (50-400 µg/mL)/IFN-α (1000 IU/mL)</td>
<td>IC₅₀ (378.71)</td>
<td>IC₅₀ (462.40-313.06)</td>
<td>IC₅₀ (445.51)</td>
<td>IC₅₀ (445.51)</td>
</tr>
<tr>
<td></td>
<td>378.71</td>
<td>462.40-313.06</td>
<td>445.51</td>
<td>445.51</td>
</tr>
<tr>
<td></td>
<td>378.71</td>
<td>462.40-313.06</td>
<td>445.51</td>
<td>445.51</td>
</tr>
</tbody>
</table>

IC₅₀ values are given in µg/mL.
Immunomodulators can be real adjuncts to established therapeutical rules offering a novel approach for the treatment of infectious diseases (31).

In the conducted experiments, the ability to reduce the cytopathic effect caused by the reference acyclovir susceptible and clinical acyclovir resistant HHV-1 strains (showing different mechanisms of resistance) was investigated. This study was focused on the question if the antiviral effect is enhanced through the addition of IFN. Biron was the first reviewer who stated clearly that IFN-α plays an important role in shaping downstream innate and adaptive immune responses to viral infections (32). Cytokine-induced antiviral mechanisms are differentially expressed in different cell lines. The degree of inhibition of HHV-1 replication in vitro depends on the cell line, and an examined strain (22).

The available evidences indicate that IFN severely inhibits the replication of several viruses in vitro. Some studies indicate that replication of HHV-1 is poorly reduced in cultured cells. However, results of in vivo studies with IFN receptor-null mice and HHV-1 strains illustrated the importance of IFN-α in controlling early stages of infection in animals (22, 34). Mossman mentioned that the absence of interferon receptors resulted in disseminated infection and severe disease. For this reason further in vivo drug interaction studies based on in vitro evaluation are necessary.

So far, a combination of IFN-α and isoprinosine has brought positive results in the treatment of subacute sclerosis panencephalitis (SSPE). SSPE is a persistent and chronic encephalitis secondary to measles virus infection that causes widespread demyelination of the central nervous system. Such combined therapy is still recommended because of theoretical synergistic effects (35). Like Mossman et al. and Kuczker et al. we have observed that the degree of inhibition of viral replication in vitro depends on the cell line, and examined strain (10, 22). IP at a concentration of 400 µg/mL exerts the strongest inhibitory effect on the replication of HHV-1MC in HEL 299 cell line. It seems that the best choice for an in vitro susceptibility testing can be the primary human lung fibroblasts.

During the past decades, cytokine-based therapies have been developed. To date, multiple cytokines with Th1-stimulating properties, such as IFN-α, IL-2 and IL-12, have been evaluated, alone or in combination for the treatment of bladder cancer (30, 36). Our studies have confirmed the observations made by other authors that the effect of interferon can be augmented after the simultaneous addition of other drugs (31, 35-38).

The enhanced antiviral activity was found when infected cells were treated with IFN-α and IP simultaneously. When IFN-α was added to HHV-1-infected cells alone, only a weak antiviral activity was detected even the concentration was as high as 1000 IU/mL. Results are statistically significant only for HHV-1MC treated simultaneously with maximal doses of IP and INF-α in HEL-299 culture.

The IC₅₀ values were most strongly reduced when IFN-α (1000 IU/mL) and IP were added. The most potent inhibitory effect on viral replication was observed when combination of maximal doses of IP and IFN-α to the HHV-1MC were used. In this case, IC₅₀ has been reduced by 26.3 and 49.8%, respectively, in HEL 299 and HEP-2 cell lines. The synergistic effect of IFN-α in combination with other drug is well known. Falzarano et al. identified a potential therapeutic approach against nCoV (novel β coronavirus) isolate combining IFN-α and ribavirin. Either treatment alone reduced virus replication by at least 1 log or as much as 4 logs in susceptible cell lines. Moreover, when both agents were combined, the efficacy was reached at lower concentrations (37). Mosa et al. suggest that combination of interferon and ribavirin should be considered in immunocompromised patients and probably also in cases of diffuse and/or complicated papillomatosis unresponsive to first- and second-line treatments (38).

It is well known that acyclovir is very effective in suppressing HHV-1 infections in the early stages of disease if the strain is ACV-sensitive (1, 6, 39). Inosine pranobex enhances T cell proliferation and activity and is also approved for treatment of HHV-1 and HHV-2 infections, though it is significantly less active than other more traditional antiherpes drugs.

Increased understanding of the antiviral immune mechanisms and the limited number of drugs which have been approved for medical use against HHV in the last few decades encourage to supplement HHV-infected patients with isoprinosine and passive immunotherapy. Immunotherapy may be especially beneficial in patients infected with viral strains resistant to currently known antiviral drugs.

REFERENCES


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Reactive oxygen and nitrogen species are commonly found in the environment. On the one hand, they are derived from many industrial processes, while on the other, they are the products of metabolism occurring in living organisms. However, most free radicals are generated inside cells rather than in the environment. The concentration of free radicals is tightly controlled by a group of compounds known as antioxidants. The human body has many antioxidant systems, including enzymes (catalase, superoxide dismutase), small molecules (glutathione, protein) and vitamins (α-tocopherol, ascorbic acid). These natural defenses of the body can be damaged by various factors. An imbalance in antioxidant equilibrium leads to oxidative stress, which contributes to the aging of the organism (1), the development of cancer (2) and neurodegenerative disorders (3). Therefore, it is important to search for compounds with antioxidant activity which may help to maintain homeostasis in living cells.

In our study, we investigated an isoquinoline alkaloid, 6,7-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid (1), and its derivatives. The isoquinoline alkaloid 1 is a secondary metabolite produced by the actinomycete strain Streptomyces sp. 8812, which was isolated from Brazilian jungle soil. Compound 1 inhibits the growth of Gram-positive and Gram-negative bacteria (4). In our previous studies, alkaloid 1 was obtained and purified from Streptomyces sp. 8812 fermentation broth, and its chemical structure was determined, based on physicochemical and spectroscopic NMR investigations. The synthesis of this compound was undertaken and it resulted in NMR data that fully agree with the present analysis (5).

To improve the biological features of 1, a series of its derivatives (compounds La-m, Table 1) was synthesized and their biological activities were examined. Alkaloid 1 was also synthesized and its biological activities were evaluated. The antioxidant properties of the obtained compounds were tested against several different radicals (2,2-diphenyl-1-picrylhydrazyl (DPPH*), 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS**)), superoxide...
Table 1. Tested molecules and their intracellular antioxidant activity against reactive oxygen and nitrogen species. Values of the effective concentration (EC<sub>50</sub>) are expressed as µM.

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; NO•</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Log P</th>
<th>LLE NO•</th>
<th>LLE H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,7-Dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>87.21</td>
<td>93.02</td>
<td>0.62</td>
<td>3.44</td>
<td>3.41</td>
</tr>
<tr>
<td>7-Bromo-8-hydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>687.65</td>
<td>n.a.</td>
<td>1.61</td>
<td>1.55</td>
<td>-</td>
</tr>
<tr>
<td>6,8-Dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>591.05</td>
<td>905.91</td>
<td>0.62</td>
<td>2.61</td>
<td>2.43</td>
</tr>
<tr>
<td>6,7-Dihydroxy-8-iodo-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>25.62</td>
<td>28.98</td>
<td>1.17</td>
<td>3.41</td>
<td>3.37</td>
</tr>
<tr>
<td>8-Bromo-5,6-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>n.a.</td>
<td>475.45</td>
<td>1.23</td>
<td>-</td>
<td>2.10</td>
</tr>
<tr>
<td>8-Bromo-6,7-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>171.65</td>
<td>43.54</td>
<td>1.23</td>
<td>2.54</td>
<td>3.13</td>
</tr>
<tr>
<td>8-Chloro-6,7-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>39.66</td>
<td>164.28</td>
<td>1.09</td>
<td>3.31</td>
<td>2.69</td>
</tr>
<tr>
<td>6,8-Dihydroxy-7-methoxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>652.20</td>
<td>273.05</td>
<td>0.47</td>
<td>2.72</td>
<td>3.10</td>
</tr>
<tr>
<td>7-Bromo-6,8-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>702.77</td>
<td>327.51</td>
<td>1.23</td>
<td>1.92</td>
<td>2.26</td>
</tr>
<tr>
<td>5-Chloro-6,8-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>266.26</td>
<td>358.14</td>
<td>1.09</td>
<td>2.48</td>
<td>2.35</td>
</tr>
<tr>
<td>5-Chloro-6,7-dihydroxy-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>56.63</td>
<td>124.75</td>
<td>1.09</td>
<td>3.15</td>
<td>2.81</td>
</tr>
<tr>
<td>7,8-Dihydroxy-4,5-dihydro-3H-benzo[c]azepine-3-carboxylic acid</td>
<td>80.28</td>
<td>124.75</td>
<td>0.98</td>
<td>3.12</td>
<td>2.93</td>
</tr>
<tr>
<td>6,7-Dihydroxy-3-methyl-3,4-dihydroisoquinoline-3-carboxylic acid</td>
<td>47.25</td>
<td>101.58</td>
<td>0.99</td>
<td>3.34</td>
<td>3.00</td>
</tr>
<tr>
<td>(4S)-1-Chloro-7,8-dihydroxy-4-methyl-2-oxo-2,4,5,9b-tetrahydro-1H-azeto[2,1-a]isoquinoline-4-carboxylic acid</td>
<td>139.12</td>
<td>66.54</td>
<td>1.56</td>
<td>2.29</td>
<td>2.61</td>
</tr>
<tr>
<td>6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid</td>
<td>55.80</td>
<td>51.88</td>
<td>2.68</td>
<td>1.57</td>
<td>1.60</td>
</tr>
</tbody>
</table>

- not active
Intracellular antioxidant activity of a Streptomyces sp. 8812 secondary metabolite... 647

anion and nitric oxide (6)). Then, compounds possessing properties superior or comparable to the standard (ascorbic acid or Trolox) were chosen and were examined in live cells. In vivo antioxidant activity should be tested because some compounds scavenge free radicals (e.g., ABTS**+, DPPH**, superoxide radical) in vitro but do not exhibit antioxidant activity in living cells. Direct evaluation of the ability of these chemical compounds to penetrate the cell membrane and inhibit reactive nitrogen and oxygen species is possible with the use of specific fluorescent probes such as 2',7'-dichlorofluorescein diacetate (7).

EXPERIMENTAL

Chemicals

Compound 1 was obtained from L-DOPA according to a published procedure (5). Derivatives of isoquinoline alkaloid 1 were synthesized in a multistep procedure using the Bischler-Napieralski reaction as a cyclization step according to methods precisely described previously (6). Streptomycin, penicillin, DMSO, sodium nitroprusside, 2',7'-dichlorofluorescein diacetate and fetal bovine serum were obtained from Sigma-Aldrich (Poznań, Poland). Hydrogen peroxide was supplied by Avantor Performance Materials Poland S.A. (Gliwice, Poland). Eagle’s minimum essential medium (EMEM) was produced by BIOMED-LUBLIN Serum and Vaccine Production Plant (Lublin, Poland). All other reagents were of standard analytical grade.

Vero culture

Vero cells (fibroblasts from the kidney of African green monkey, ATCC® CCL-81™) were cultured in EMEM with 10% heat-inactivated fetal calf serum, streptomycin (100 µg/mL) and penicillin (100 unit/mL) at 37°C in an atmosphere of 5% CO₂ (8). At 16-18 h prior to determination, cells were settled in a 96-well black Nunclon Delta Surface (Thermo Scientific, USA) microplate (100 µL culture, 1 × 10⁵ cells/mL in the well) to obtain a monolayer.

Intracellular antioxidant activity measurement

The amount of 5.5 µL of increasing concentrations of the tested compounds dissolved in DMSO (final concentrations: 2.9, 5.9, 11.8, 23.5, 47.0, 70.5, 94.0 and 188.0 µM) were added to the appropriate wells in a microplate with settled Vero cells and incubated for 30 min at 37°C. Then, to each well, 5.5 µL of H₂O₂ solution (final concentration: 1 mM) was added and incubated for another 30 min at 37°C. Then, to all wells, 6 µL of a solution of 2',7'-dichlorofluorescein diacetate (DCFH-DA, final concentration: 5 µg/mL) was added. The fluorescence (ex. 485 nm, em. 520 nm) was read on a BMG LABTECH FLUOstar Omega microplate reader. Trolox was used as a standard.

Reactive nitrogen species were generated from sodium nitroprusside (9). Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide; therefore, it was dissolved in 0.1 M phosphate buffer pH 7.4. It was added to wells (5.5 µL to a final concentration 1 mM) instead of H₂O₂ solution. The remaining part of the procedure was the same as described for the scavenging of H₂O₂.

The intracellular antioxidant activity of the tested compounds (percent of scavenged reactive oxygen or nitrogen) was calculated according to the formula:

\[ \frac{1 - (F_1 - F_0)}{F_0} \times 100 \]

where F₀ is the fluorescence of the cells treated with the reactive form of oxygen or nitrogen species, and F₁ is the fluorescence of cells treated with the reactive forms of oxygen or nitrogen and the tested compound. All samples were replicated three times.

Calculations

In order to better understand the overall properties of the described compounds, the theoretical prediction of ADME properties (octanol-water partition coefficient (log P), aqueous solubility (log S), number of hydrogen bond donors (HBD) and acceptors (HBA), molecular weight (m.w.)) was carried out with the use of ICM Pro (Molsoft L.L.C.). Moreover, the polar surface area (PSA), number of rotatable bonds (no. of RotB) and volume (vol) for compounds 1 and 1a-m were determined. Based on the estimated theoretical log P value and IC₅₀ value for the tested compounds, the lipophilic ligand efficiency (LLE) was calculated according to the equation (10):

\[ \text{LLE} = \frac{\text{pIC}_{50} - \text{cLog P}}{\text{IC}_{50}} \]

The same parameters were calculated for the standard (Trolox) and compared to the tested compounds.

Statistical analysis is expressed as the means of three measurements. The results were statistically significant when the p value was less than 0.05. The amount of compound needed to scavenge the free radical concentration by 50% (IC₅₀) was calculated by the linear regression method using R 3.0.1 statistical software (R Core Team (2013) (11)).
RESULTS

Tested compounds had variable antioxidant activity within Vero cells and showed diverse log P (0.47 - 1.61) and LLE values (1.55 - 3.44, Table 1). Compound 1d did not scavenge NO• free radicals, whereas 1a did not scavenge H2O2. Four compounds – 1c, 1f, 1l and 1j – inactivated the reactive form of nitrogen more efficiently than Trolox (EC50 = 55.80 µM). The EC50 values were 25.62, 39.66, 47.25 and 56.63 µM, respectively (Fig. 1). On the other hand, only two compounds, 1c and 1e, were more potent scavengers of reactive oxygen species (EC50 = 28.98 and 43.54 µM, respectively) than Trolox (EC50 = 51.88 µM, Fig. 2). The most active derivative of 1 was compound 1c containing an iodine atom at position 8.

Compounds evaluated in the study showed PSA values in the range of 53.63 to 77.90 Å² (Table 3). In addition, the molecular volume values were in the range of 173.96 to 282.23 Å³. The tested compounds had one or two rotatable bonds and one aromatic ring.

The results show that compounds 1 and 1a-m obeyed the Lipinski rule of five (MW = 500, HBD =

Figure 1. Intracellular antioxidant effectiveness against nitric oxide

Figure 2. Intracellular antioxidant effectiveness against hydrogen peroxide
Intracellular antioxidant activity of a Streptomyces sp. 8812 secondary metabolite...

The molecular weight of compounds was between 207.05 and 332.95 Da; they possessed two or three H-bond donors and four to six H-bond acceptors. Trolox exhibited very similar values to the study compounds for all calculated parameters.

### DISCUSSION AND CONCLUSIONS

Our results demonstrate that the alkaloid 1 and a few synthetic derivatives (hydroxyl-, methyl- and halogeno-substituted 3,4-dihydroisoquinoline-3-carboxylic acids, the 2-oxo-1,4,5,9b-tetrahydro-2H-azeto[2,1-al]isoquinoline analogue and the 4,5-dihydro-3H-2-benzazepine analogue) have the ability to penetrate the cell membrane of living cells. In cells, these compounds may maintain the redox equilibrium, and thus contribute to the protection of DNA and other cellular structures from the damaging effects of reactive oxygen and nitrogen species.

The polar surface area values of the tested compounds were found to be compatible with those required to cross membranes. The PSA is defined as the area occupied by nitrogen and oxygen atoms as well as hydrogen atoms attached to these heteroatoms (14). It is a predictor of passive intestinal...
absorption of a drug in the human body (15). It is assumed that drugs are completely absorbed when their PSA values are equal to or less than 60 Å², whereas only 10% absorption is estimated when PSA values are greater than 140 Å² (14). Therefore, the tested compounds exhibited adequate PSA values to pass through cell membranes.

Another predictor of bioavailability is the number of rotatable bonds. Compounds 1 and 1a-m possess at most only one rotatable bond (compound 1g has two rotatable bonds). According to a study by Veber et al., no. of RotB = 10, tPSA = 140 Å² and HBA and HBD = 12 are predictors of good oral bioavailability (16).

The results show that all Lipinski rule of five descriptors (MW, HBD, HBA and log P) for compounds 1 and 1a-m had moderate values. So, they will likely have favorable pharmacokinetics and be well absorbable.

A positive correlation was also noted between the EC50 against NO* and the EC50 against H2O2 (n = 13, r = 0.70, p < 0.05; Table 3) was observed. This might suggest that compounds are similarly effective in scavenging nitrogen as well as oxygen reactive species inside cells (Table 1). In other words, if the compounds have antioxidant properties, they scavenge the majority of free radicals.

A positive correlation was also noted between the EC50 against NO* and H2O2 and log S, PSA, no. of RotB, HBA and HBD (Table 3). Possibly, compounds with lower values of log S, PSA, no. of RotB, HBA and HBD show better scavenging activity against oxygen and nitrogen reactive species.

Moreover, a negative but non-significant correlation was noted between the EC50 against NO* and H2O2, and molecular volume and the number of rings. More potent NO* and H2O2 scavengers are compounds that have higher volume values as well as have more rings in the structure (Table 1).

Similarly, a non-significant negative correlation between log P and EC50 as well as between the LLE and the EC50 value of the tested compounds was observed (Table 3). Generally, compounds with higher lipophilicity are better at scavenging free radicals inside cells, but there might be a few explanations for the weakness of the statistical significance. Log P is not the best determinant of lipophilicity for compounds because it only correctly describes the partition coefficient of uncharged molecules. The majority of drugs are ionized at the pH of the different compartments of the cell, therefore log P is not an appropriate predictor of a compound’s characteristics. Some compounds, in spite of possessing a low log P value, do not penetrate into cells through the cell membrane, or the molecules pass into cells but intracellular biochemical processes destroy their activity. It might be supposed that some compounds utilize some transmembrane canals or other proteins rather than simple diffusion to pass through the membrane barrier. Moreover, some compounds do not react with the fluorescent antioxidant activity indicator (17).

It has been shown that some of the evaluated compounds had antioxidant activity comparable to or better than that of Trolox. The tested molecules possessed antioxidant activity in vitro (6), and some of them exhibited antioxidant activity in vivo. The analysis of molecular descriptors revealed that these compounds should have favorable pharmacokinetics and are drug-like (18). Therefore, the obtained molecules can be used as scaffolds for the design of new therapeutic agents to protect against oxidative stress.

Acknowledgments

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REFERENCES


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Paracetamol (acetaminophen) is commonly used as a drug of choice for treatment of pain and fever. Unlike non-steroidal anti-inflammatory drugs (NSAIDs) it does not cause gastrointestinal damage or untoward cardioireal effects, however cutaneous adverse effects have been reported. It is known that paracetamol binds to melanin biopolymers, but the relation between the affinity of this drug to melanin and its toxicity is not documented. The aim of this work was to examine the impact of paracetamol on melanogenesis in cultured human normal epidermal melanocytes (HEMn-DP). The effect of paracetamol on cell viability was determined by WST-1 assay, melanin content and tyrosinase activity were measured spectrophotometrically. It has been demonstrated that paracetamol induced concentration-dependent loss in melanocytes viability. The value of EC₅₀ was found to be ~ 20.0 mM. The analyzed drug inhibited melanin biosynthesis in a concentration-dependent manner by decreasing the melanin content as well as the tyrosinase activity. The demonstrated inhibitory effect of paracetamol on melanization process in normal epidermal melanocytes in vitro may explain the potential role of melanin biopolymer in the mechanisms of undesirable side effects of this drug in vivo, as a result of its accumulation in pigmented tissues.

Keywords: paracetamol, melanocytes, viability, melanization, tyrosinase
concentrations. On the other hand, the long-term exposure may build up high levels of noxious chemicals stored in the melanin, which ultimately may cause degeneration in melanin-containing cells and secondary lesions in surrounding tissues (20).

Previously, we have documented that various drugs suppressed melanin biosynthesis in human light (ciprofloxacin (21), lomefloxacin (22), amikacin (23), netilmicin (25), streptomycin (26), nicotine (27), gentamicin (28)) and dark (nicotine (29), chlorpromazine (30), gentamicin (28)) pigmented melanocytes. Moreover, we have also documented that paracetamol forms stable complexes with model synthetic melanin (31). The effect of paracetamol on melanin biosynthesis in melanocytes has not been studied so far. This work is a continuation of our earlier studies and may be significant for the assessment of toxic reactions produced by this drug in human organism.

The aim of this work was to examine the effect of paracetamol on melanization process in cultured human epidermal melanocytes (HEMn-DP).

EXPERIMENTAL

Chemicals

Phosphated-buffered saline (PBS), 3,4-dihydroxy-L-phenylalanine (L-DOPA), synthetic melanin and amphotericin B were purchased from Sigma-Aldrich Inc. (USA). Paracetamol was obtained in the form of solution — Perfalgan (10 mg/1 mL) from Bristol-Meyers Squibb (Poland). Neomycin sulfate was obtained from Amara (Poland). Penicillin was acquired from Polfa Tarchomin (Poland). Growth medium M-254 and human melanocyte growth supplement-2 (HMGS-2) were obtained from Cascade Biologics (UK). Trypsin/EDTA was obtained from Cytogen (Poland). Cell Proliferation Reagent WST-1 was purchased from Roche GmbH (Germany). The remaining chemicals were produced by POCH S.A. (Poland).

Cell culture

The normal human epidermal melanocytes, neonatal, dark pigmented (HEMn-DP) from Cascade Biologics (UK) were grown according to the manufacturer’s instruction. The cells were cultured in M-254 basal medium supplemented with HMGS-2, penicillin (100 U/mL), neomycin (10 µg/mL), and amphotericin B (0.25 µg/mL) at 37°C in 5% CO₂. All experiments were performed using cells in the passages 5–8.

Cell viability assay

The viability of melanocytes was evaluated by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay. WST-1 is a water soluble tetrazolium

[Graph: Figure 1. The effect of paracetamol on viability of melanocytes. Cells were treated with various paracetamol concentrations (0.001–20.0 mM) and examined by WST-1 assay. Data are expressed as % of cell viability. Mean values ± S.E.M. from three independent experiments performed in triplicate are presented. ** p < 0.01 vs. the control samples]
solv, the rate of WST-1 cleavage by mitochondrial dehydrogenases correlates with the number of viable cells. In brief, 5000 cells per well were placed in a 96-well microplate in a supplemented M-254 growth medium and incubated at 37°C and 5% CO₂ for 48 h. Then, the medium was removed and cells were treated with paracetamol solutions in a concentration range from 0.001 to 20.0 mM. After 21-h incubation, 10 µL of WST-1 was added to 100 µL of culture medium in each well, and the incubation was continued for three hours. The absorbance of the samples was measured at 440 nm with a reference wavelength of 650 nm, against the controls (the same cells but not treated with paracetamol) using a microplate reader UVM 340 (Biogenet, Poland). The controls were normalized to 100% for each assay and treatments were expressed as the percentage of the controls.

Measurement of melanin content

The melanocytes were seeded in T-25 flasks at a density of 1 × 10⁵ cells per flask. Paracetamol treatment in a concentration range from 0.2 to 20.0 mM began 48 h after seeding. After 24 h of incubation, the cells were detached with trypsin/EDTA. Cell pellets were placed into Eppendorf tubes, dissolved in 100 µL of 1 M NaOH at 80°C for 1 h, and then centrifuged for 20 min at 16,000 × g. The supernatants were placed into a 96-well microplate and absorbance was measured at 405 nm - a wavelength at which melanin absorbs light (32). Melanin content in paracetamol-treated cells was expressed as the percentage of the controls (untreated melanocytes).

Tyrosinase activity assay

Tyrosinase activity in HEMn-DP cells was determined by measuring the rate of oxidation of L-DOPA to DOPAchrome, according to the method described earlier (21, 28). The cells were cultured at a density of 1 × 10⁵ cells in T-25 flasks for 48 h. After 24-h incubation with paracetamol (concentration range from 0.2 to 20.0 mM), cells were washed three times with PBS, lysed and clarified by centrifugation at 10,000 × g for 5 min. A tyrosinase substrate L-DOPA (2 mg/mL) was prepared in the same lysis phosphate buffer. Hundred microliters of each lysate was put in a 96-well plate, and the enzymatic assay was initiated by the addition of 40 µL of L-DOPA solution at 37°C. Absorbance was measured every 10 min for at least 1.5 h at 475 nm using a microplate reader. Tyrosinase activity was expressed as the percentage of the controls (untreated melanocytes).

Statistical analysis

In all experiments, mean values of at least three separate experiments performed in nine replications (n = 9) ± standard error of the mean (S.E.M.) were calculated. The results were analyzed statistically using GraphPad Prism 6.01 software by means of one-way ANOVA and Tukey’s multiple comparison test. A value of p < 0.05 (*) or p < 0.01 (**), obtained by comparing the data with those for control (cells without paracetamol), was considered statistically significant.

RESULTS

To assess the influence of paracetamol on the viability of melanocytes, cells were treated with paracetamol in a range of concentrations from 0.001 mM to 20.0 mM for 24 h (Fig. 1). The cell viability was determined by the WST-1 test assay. For paracetamol concentrations from 0.001 mM to 1.0 mM, changes were not statistically significant. After treatment of cells with 5.0, 7.5, 10.0 and 20.0 mM of paracetamol, the loss in cell viability by 10.9, 11.6, 34.6 and 48.7%, respectively, was observed. The value of EC₅₀ (i.e., the concentration of a drug that produces loss in cell viability by 50%) was ~ 20.0 mM.

The effectiveness of melanization process was estimated by measuring the melanin content and cellular tyrosinase activity in melanocytes treated with paracetamol in EC₅₀ concentration as well as 10-fold and 100-fold lower concentrations, for 24 h. After determining a calibration curve, the melanin content per cell was determined as 55.2±58.0 pg/cell for melanocytes treated with the paracetamol and 60.8 ± 1.7 pg/cell for a control samples. The obtained results, recalculated for the culture (1 × 10⁵ cells), were finally expressed as a percentage of the controls (Fig. 2). Paracetamol in concentration of 0.2 mM had no effect on melanin content. In cells treated with paracetamol in concentrations of 2.0 and 20.0 mM for 24 h, melanin production decreased by about 7 to 11%, respectively.

Tyrosinase activity in HEMn-DP cells treated with paracetamol also decreased in a manner correlating well with the inhibitory effect on melanin formation (Fig. 3). After 24-h incubation with paracetamol, tyrosinase activity was suppressed to 87% at 0.2 mM, 83% at 2.0 mM and to 80% at 20.0 mM, when compared with the controls.

DISCUSSION

Paracetamol is extensively used for the treatment of pain and fever. Moreover, it is a drug of
choice when application of non-steroidal anti-inflammatory drugs are contraindicated, e.g., in the case of gastric ulcers, hypersensitivity to aspirin, impairments in blood coagulation, in pregnant women, breastfeeding mothers and children with fever accompanying a disease (2).

Previously, we have documented that paracetamol forms stable complexes with synthetic DOPA-melanin characterized by the association constants $K_1 \approx 4.5 \times 10^5 \text{ M}^{-1}$ and $K_2 \approx 2.45 \times 10^4 \text{ M}^{-1}$. The total number of binding sites was estimated to be 8.9 (nmol drug/mg melanin) (31).

The ability of melanin to bind many drugs and chemical substances may have concurrently beneficial or negative effects on the organism. This interaction protects melanocytes and other cells against excessive exposure to potentially dangerous compounds through their previous accumulation and further elimination in non-toxic concentrations. However, a chronic exposition to xenobiotics with high affinity for melanin may cause degeneration of cells containing melanin. It is believed that the process of drug-induced damages of tissues containing melanin takes place when the detoxifying capacities of melanin are exhausted (20, 33). This effect is mainly connected with high dose and/or long-term exposure to drugs. The onset of the adverse effects may be delayed, and the entire manifestation of the lesions may occur even years after cessation of the offending substance (33).

Based on the previous study concerning the interaction of paracetamol with synthetic melanin (31), it can be concluded that paracetamol binds to melanin strongly but in low amounts. Thus, the unbound drug is able to cause the skin adverse reactions in vivo, e.g., drug eruption (4), urticaria, rashes or blisters (7). The most interesting side effect is the ability of paracetamol to cause eczema at school age children which were treated with this drug at early childhood (13, 14, 34, 35). It is an example of cutaneous adverse effect, which may be connected with delayed or slow release and dislocation of paracetamol from complexes with melanin to circulatory system.

In the present study, the effect of paracetamol on melanocytes viability, as well as on melanization process in pigmented cells was analyzed. We used the culture of normal human melanocytes HEMn-DP as an in vitro experimental model system.

The obtained results have shown that paracetamol in a range of concentrations from 0.001 mM to 1.0 mM had no effect on cell viability (Fig. 1).

![Figure 2](image-url)

Figure 2. The effect of paracetamol on melanin content in melanocytes. Cells were cultured with 0.2, 2.0 or 20.0 mM of paracetamol for 24 h and melanin content was measured as described in Material and Methods. Results are expressed as percentages of the control samples. Data are the mean ± S.E.M. of at least three independent experiments performed in triplicate. * $p < 0.05$ vs. the control samples; ** $p < 0.01$ vs. the control samples.
Effect of paracetamol on melanization process in human epidermal melanocytes

Higher concentrations of the drug above 1.0 mM, resulted in a concentration-dependent loss in melanocytes viability. The value of EC\textsubscript{50} was \( \approx 20.0 \) mM.

The changes of melanin content and tyrosinase activity in melanocytes were analyzed for paracetamol concentrations of 0.2 mM, 2.0 mM and 20.0 mM. The obtained results have shown that paracetamol in concentration of 0.2 mM does not significantly affect the melanin content in melanocytes (Fig. 2). Treatment of cells with paracetamol in concentrations of 2.0 mM and 20.0 mM caused decrease in melanin content to 93% and 89%, respectively.

The melanization process in melanocytes was also determined by the activity of tyrosinase, a key enzyme responsible for the activation of the pigmentation machinery. This enzyme can be inhibited by different substances and drugs, e.g., ketoprofen (36), gentamicin (28), kanamycin (24). In our study we have demonstrated that paracetamol in concentrations of 0.2 mM, 2.0 mM and 20.0 mM suppressed tyrosinase activity to 87, 83 and 80%, respectively (Fig. 3), what may explain the observed decrease in melanin content in melanocytes.

To summarize, our study shows that paracetamol in higher concentrations (above 1.0 mM) reduces melanocytes viability. This drug also inhibits tyrosinase activity leading to reduction in melanin content.

CONCLUSION

The current study has shown that paracetamol inhibits melanization process in normal human epidermal melanocytes \textit{in vitro}, what may be a reason for the adverse skin reactions observed \textit{in vivo} (2, 5, 6). These side effects may be explained by the demonstrated inhibition of tyrosinase activity and decrease in melanin content in melanocytes treated with paracetamol. Thus, melanin is not able to protect melanocytes and surrounding tissues against high concentrations of paracetamol.

Acknowledgments

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REFERENCES


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Doxorubicin (DOX) as an effective chemotherapy drug has serious side effects such as dilated cardiomyopathy and congestive heart failure (1). Therefore, usage of this drug must be limited. The mechanisms of DOX-induced cardiotoxicity are not completely understood, but most evidences indicate that the generation of reactive oxygen species (ROS) is involved (2). Increased level of ROS leads to protein and lipid peroxidation, DNA damage and irreversible cell damage (3, 4). ROS can directly impair contractile function, activate hypertrophy signaling pathways, stimulate cardiac fibroblast proliferation and induce extracellular matrix remodeling (5, 6). Excessive ROS also cause accumulation of intracellular Ca\(^{2+}\) in cardiac cells, which increases the mitochondrial permeability and leads to the release of cytochrome c into the cytoplasm and the following apoptotic cascades (7). Interestingly, some natural foods have been reported to contain substantial amounts of antioxidants and free radical scavenging agents. These compounds diminish some side effects of chemotherapeutic agents on normal cells by reducing their genotoxicity (2). H9C2 myoblasts, a rat embryonic cell line, which has the ability to differentiate between a skeletal or cardiac muscle phenotype, can be instrumental in understanding DOX cytotoxicity in different stages (8). The recent studies have shown that some of the herbs act against oxidative injury-related cardiotoxicity. 

*Lactuca serriola* L. (Compositae) is known by several names such as Prickly lettuce, jagged lettuce, Kahu and Khas (9). It is native to Himalaya, Siberia, and Atlantic areas (9) but cultivated also in temperate lands of Europe, India, Pakistan, and Iran (9). In traditional medicine, this plant is applied as a sedative, hypnotic, expectorant, coughs suppressant, purgative, diuretic, antiseptic, vasorelaxant, and antispasmodic (9). The plant contains vitamins, β-carotene, and iron. The obtained extract of this plant is composed of lactucone, lactucin, and lactucic acids (9). There are alkaloids, the bitter substance of lettuce, oxalic acid, lactucopinic, and sesquiterpenes esters in seeds. The antipyretic activity is related to alkaloid and lactucin, whereas isolated triterpenoid

**PROTECTIVE EFFECT OF *LACTUCA SERRIOLA* ON DOXORUBICIN-INDUCED TOXICITY IN H9C2 CELLS**

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**Abstract:** The use of doxorubicin (DOX) is limited by its dose-dependency because of its cardiotoxicity. Reactive oxygen species (ROS) play an important role in the pathological process. The aim of this study is to evaluate the protective effect of *Lactuca serriola* against DOX-induced apoptosis and death in H9C2 cells. The cells were incubated with different concentrations of extract for 4 h which continued in the presence or absence of 5 µM doxorubicin for 24 h. Cell viability, apoptotic induction and the level of apoptotic proteins were determined by using MTT, PI and immunoblotting assays, respectively. The level of lipid peroxidation was measured by fluorimetric method. DOX significantly decreased cell viability which was accompanied by an increase in ROS production and lipid peroxidation. Pretreatment with *Lactuca serriola* increased the viability of cardiomyocytes and could decrease lipid peroxidation. Also, *Lactuca serriola* inhibited the reduction of anti-apoptotic Bcl-2 protein and elevation of apoptotic Bax and caspase-3 proteins. In conclusion, *Lactuca serriola* exerts protective effect against oxidative stress-induced cardiomyocytes damage. Therefore, it has the potential to be used as cardioprotective agent by the patients with cardiovascular diseases.

**Keywords:** *Lactuca serriola*, H9c2, doxorubicin, apoptosis

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saponin possesses antibacterial activity (9). The studies have shown that plant has analgesic, anti-inflammatory, and antioxidant activities due to totally high phenolic contents. The antioxidant activity scavenges the free radicals and reduces oxidative stress (9). In this study, the protective effect of hydro-alcoholic extract of *Lactuca serriola* on DOX-induced cardiotoxicity was evaluated.

**MATERIALS AND METHODS**

**Reagents**

*Lactuca serriola* was obtained from local market, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), thioarbituric acid (TBA), bixin-chonic acid protein assay kit and protease inhibitor cocktail, propidium iodide (PI), sodium citrate and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). High-glucose Dulbecco’s modified Eagles medium (DMEM), penicillin-streptomycin and fetal bovine serum were purchased from Gibco. Trichloroacetic acid (TCA) and malondialdehyde bis-(dimethyl acetal) (MDA) were obtained from Merck (Darmstadt, Germany). H9C2 cells were obtained from Pasteur Institute (Tehran, Iran). The Bax, Bcl-2 and caspase 3 antibodies were purchased from Cell Signaling Technology Inc. Doxorubicin, manufactured by EBWE company, was purchased from a pharmacy.

**Preparation of extracts**

The aerial parts of *Lactuca Serriola* were collected from the garden of Ferdowsi University of Mashhad, Mashhad, Iran. The plant sample was identified at the herbarium of School of Pharmacy (Mashhad University of Medical Sciences, Mashhad, Iran) and a specimen voucher (12829) was deposited in this institute. The aerial parts of lettuce were dried, powdered and 50 g of this powder was subjected to extraction with 70% ethanol in a Soxhlet apparatus for 48 h. The hydro-alcoholic extract was then dried on a water bath and kept frozen in less than -18°C for the following use. The yield of extract was 19% w/w. The extract was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 mg/mL before being used in cytotoxicity and apoptosis assays.

**Cell culture**

H9c2 cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. For the experiments, they were seeded in 96-well and 24-well culture plates for MTT and MDA assays, respectively. For apoptosis assay, cells were seeded at 100,000/well in a 24-well plate. All treatments were carried out in triplicate. The cells were pretreated with extract (6-200 µg/mL) for 4 h and then incubation was continued in the presence of the extract with 5 µM doxorubicin for 24 h.

**Cell viability**

The cell viability was determined using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay as described previously (10, 11). Briefly, MTT solution in phosphate-buffered saline (5 mg/mL), was added to each well at final concentration of 0.05%. After 3 h, the formazan precipitate was dissolved in DMSO. The absorbance at 570 and 620 nm (background) was measured using a StatFAX303 plate reader.

**Apoptosis**

Apoptotic cells were detected by using PI staining of the treated cells followed by flow cytometry to detect the so-called sub-G1 peak (12). Briefly, H9C2 cells were cultured overnight in a 24-well plate and pretreated by *Lactuca serriola* for 4 h and then treated with DOX for 24 h. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 500 µL of a hypotonic buffer (50 µg/mL PI in 0.1% sodium citrate plus 0.1% Triton X-100).

**Lipid peroxidation assay**

The level of lipid peroxidation was estimated by measuring MDA which is the end product of lipid peroxidation. At the end of incubation, the cells were scraped and centrifuged for 30 min. Then, 400 µL of TCA (15%) and 800 µL of TBA (0.7%) were added to 500 µL of cell samples. The mixture was vortexed and then heated for 40 min in a boiling water bath. Then, 200 µL of the sample was transferred to 96-well plate and the fluorescence intensity was read with excitation/emission of 480/530 nm. The experiment was carried out in triplicate.

**Western blot analysis**

The cells were lysed using lysis buffer containing 50 mM Tris-HCl, 2 mM EDTA, 2 mM EGTA, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 0.2% w/v sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail. After centrifuging, the protein concentration of the supernatants was measured and equal amounts of protein from samples were mixed with loading buffer. Samples were sep-
Protective effect of Lactuca serriola on doxorubicin-induced toxicity.

Isolated and purified proteins were separated by electrophoresis in 12% SDS-PAGE gels and were transferred to polyvinylidene fluoride membranes. The blots were blocked with 5% skim milk for an overnight in a refrigerator. After blocking, blots were incubated with Bax, Bcl-2 and caspase 3 antibodies for 2 h at room temperature. The membrane was washed with 0.1% Tween 20 and the bound antibody was made visible using horse radish peroxidase-conjugated goat anti-rabbit secondary antibody and an enhanced chemiluminescence system. Bands were analyzed using Gel Pro Analyzer Software (Media Cybernetics).

Characterization of the extract by HPLC

The quality of hydro-alcoholic extract of Lactuca Serriola was characterized by HPLC-UV fingerprint. The chromatographic separation was carried out with a reverse-phase Waters C18 analytical column (250 x 4.6 mm, 5 µm particle size). An isocratic elution was carried out by the mobile phase of methanol : acetonitrile : water (60 : 20 : 20% v/v/v, pH 5.9 adjusted with phosphoric acid) at the flow rate of 1 mL/min. The UV detector wavelength was set at 330 nm. A sample of the extract was dissolved in distilled water and passed through 0.45 µm membrane filter. Then, 20 µL of the sample (500 µg/L) was injected into the HPLC column.

Statistical analysis

All the results were expressed as the mean ± SEM. The significance of difference was evaluated with ANOVA and Bonferroni’s test. A probability level of p < 0.05 was considered statistically significant.

RESULTS

Effect of Lactuca serriola extract on cell viability

Incubation with DOX significantly decreased cell viability to 51.3 ± 0.95% of control (p < 0.001). Pretreatment with 25, 50, 100 and 200 µg/mL of Lactuca serriola could increase the viability of H9C2 cells to 71 ± 0.43% (p < 0.01), 79 ± 4% (p < 0.001), 87 ± 3.6% (p < 0.001) and 69 ± 2% (p < 0.01) of control, respectively (Fig. 1). At the dose of 6 and 12 µg/mL, however, Lactuca serriola was not able to protect H9C2 cells against DOX-induced cytotoxicity.

Effect of Lactuca serriola on apoptotic induction

Apoptosis in H9C2 cell line was detected by flow cytometry using PI staining. Cells were pretreated for 4 h with various concentrations of the extract and exposed to DOX for 24 h. Analysis of the subG1 peak in flow cytometry histograms revealed the induction of apoptosis in cells which were treated by DOX (p < 0.001). Lactuca serriola decreased apoptotic induction significantly, at the doses of 50 µg/mL (p < 0.01), 100 µg/mL (p < 0.001) and 200 µg/mL (p < 0.05) (Fig. 2). The most reduction of apoptotic rate was at the dose of 100 µg/mL (control 2 ± 1, DOX 72 ± 2 and 100 µg/mL 28 ± 1.5).

Figure 1. Effect of Lactuca serriola on cell viability of H9C2 cells. The cells were pretreated with different concentrations of extract for 4 h then exposed to doxorubicin (DOX) for 24 h. Viability was quantitated by MTT assay. Results are the mean ± SEM (n = 3). ***p < 0.001 versus control, *p < 0.01 and **p < 0.001 versus DOX. Cont = Control
Effect of *Lactuca serriola* extract on MDA production

The level of lipid peroxidation was evaluated by measuring the level of MDA, which is the end product of lipid peroxidation. As shown in Figure 3, exposure of the cells to DOX resulted in a significant increase of MDA level (236 ± 7.9%, p < 0.001) as compared to control cells cultured in the absence of DOX (100 ± 1.3%). The content of MDA was significantly decreased in the cells pretreated with 25 µg/mL (181 ± 3.48%, p < 0.01), 50 µg/mL (168 ± 11.7%, p < 0.001), 100 µg/mL (144 ± 3.45%, p < 0.001), or 200 µg/mL (179 ± 5.6%, p < 0.01) of *Lactuca serriola*.

Effect of *Lactuca serriola* on pro-apoptotic and anti-apoptotic proteins

Incubation of H9C2 cells with DOX significantly up-regulated the expression of pro-apoptotic proteins (Bax and caspase 3) and down-regulated expression of anti-apoptotic protein (Bcl-2). Results showed that DOX increased Bax/Bcl-2 (p < 0.01)
Protective effect of *Lactuca serriola* on doxorubicin-induced toxicity...

...and caspase 3 (p < 0.01) in comparison with the control group (Fig. 4). Pretreatment with both 50 and 100 µg/mL of *Lactuca serriola* decreased the level of Bax/Bcl-2 (50 µg/mL, p < 0.05 and 100 µg/mL, p < 0.01) and caspase 3 (p < 0.01) proteins in comparison with DOX whereas the concentration of 200 µg/mL did not decrease Bax/Bcl-2 and caspase 3 significantly.

**HPLC profile of *Lactuca serriola***

A simple and reliable HPLC fingerprint had been developed for the standardization of the hydro-alcoholic extract. HPLC profile of *Lactuca serriola* was recorded under UV 330 nm. The corresponding HPLC chromatogram is presented in Figure 5. The extract revealed 5 major peaks with retention time (tR) values in the range of 1.9 to 9.7 min for 20 µL application volume (Fig. 5).

**DISCUSSION**

Doxorubicin is widely used as antineoplastic agent in the treatment of a variety of solid malignancies, such as leukemias, bladder, lung, breast and ovarian cancers. It leads to cardiotoxicity, as a result, its clinical uses are limited. Studies have shown that doxorubicin induces cardiotoxicity via elevation of ROS. Doxorubicin leads to elevation of ROS via NADPH-dependent enzymatic and a non-enzymatic pathway to generate Fe2+ doxorubicin free radicals (13). The generated doxorubicin releases radicals that cause DNA breaks and lipid peroxidation (14-16). Cardiac tissue is sensitive to oxidative damage because of its high oxidative metabolism and low antioxidant defenses in this organ compared with others (17, 18).

In the present study H9C2 cells were used as a pharmacological model to evaluate the potential cardioprotective effect of *Lactuca serriola* against doxorubicin. The results showed that *Lactuca serriola* has protective effect on H9C2 cells against DOX-induced oxidative stress. H9C2 cells are morphologically similar to immature embryonic cardiomyocytes. Considering that these cells preserve electrical and hormonal signal pathways found in adult cardiac cells (19), they are a useful model for studying oxidative stress-induced cardiomyocyte damage (20). In this model, DOX significantly increased lipid peroxidation and induced the apoptotic rate. These changes are similar to the DOX-induced deleterious effects on normal cardiac cells, which lead to the loss of cardiomyocytes viability. These changes lead to a down-regulation of anti-apoptotic protein, Bcl-2, and an up-regulation of apoptotic proteins Bax and caspase 3 which finally are accompanied by the loss of viability of cardiomyocytes. In agreement with these observations, it was demonstrated that the exposure of H9C2 cells to DOX lead to a significant increase in caspase 3 and Bax, and a considerable decrease in Bcl-2 level and cell viability (21-23). In

![Figure 3](image-url)  
**Figure 3.** The effects of *Lactuca serriola* extract on the level of MDA under doxorubicin treatment in H9C2 cells. Cells were pretreated with different concentrations of *Lactuca serriola* extract (6-200 µg/mL) for 4 h, then exposed to 5 µM doxorubicin and incubated for 24 h. Results are the means ± SEM from three independent experiments. ***p < 0.001 versus control, *p < 0.01 and **p < 0.001 versus DOX. Cont = Control
this very study, pretreatment with *Lactuca serriola* could decrease the lipid peroxidation and apoptotic rate. The antioxidant actions of *Lactuca serriola* inhibited cardiac cell death by suppression of pro-apoptotic proteins levels and increasing the level of anti-apoptotic protein.

The studies have shown that *Lactuca serriola* includes alkaloids, the bitter substance of the lettuce, oxalic acid, lactucopin (24) and sesquiterpene esters. It has analgesic, anti-inflammatory (25), and antioxidant activities. Antioxidant activities due to high total phenolic contents are proved to be efficient to release radical scavenging potential like quercetin (26, 27). Recent studies have indicated that *Lactuca serriola* has Ca\(^{2+}\) channel blocker activity, and as a result, it could act similarly to Ca\(^{2+}\) channel blockers and it can be used in cardiovascular disorders (28, 29). Diltiazem is a calcium channel blocker (30). Studies have shown that doxorubicin cytotoxicity is due to induction of calcium cycling in cardiac mitochondria. However, doxorubicin reduces the amount of Ca\(^{2+}\) accumulation in the mitochondria (31, 32). Furthermore, elevation of Ca\(^{2+}\) influx in mitochondria could lead to the loss of mitochondrial membrane potential, causing cytochrome-c to be released and subsequent caspase to be activated (33). Recent studies have shown that the elevation of Ca\(^{2+}\) levels and decrease in mitochondrial membrane potential are associated with the production of ROS and that the both steps occur prior to the induction of mitochondrial permeability transition and subsequent cell death (34). However,
Protective effect of *Lactuca serriola* on doxorubicin-induced toxicity...

Diltiazem *via* blocking the calcium channels of H9C2 cells significantly reduce the influx of extracellular Ca²⁺, which decreases the detrimental effects of doxorubicin (35). As a result, protective effect of *Lactuca serriola* can be related to antioxidant activity and Ca²⁺ channel blocker. It needs more investigation to understand the accuracy of this mechanism.

In the present chromatographic technique, in order to obtain a good resolution within a short analysis time, the composition of mobile phase was optimized. Acidic mobile phase was used in order to suppress the ionization of phenolic hydroxyl groups. This acidification was beneficial, leading to good peak separation and better peak shape. Various mobile phase compositions were evaluated. Methanol, acetonitrile and water containing little amount of phosphoric acid were chosen as the mobile phase, because all the peak components could be resolved under this condition. The HPLC fingerprint showed high stability and reproducibility, and thus, it could be used for the quality control of the hydro-alcoholic extract and *Lactuca* products.

**CONCLUSION**

In conclusion, our data demonstrated that *Lactuca serriola* exerts protective effect against DOX induced toxicity in cardiomyocytes. This effect is mediated by reducing oxidative stress and inhibiting of apoptotic pathways. Therefore, *Lactuca serriola* has the potential to be administrated as a cardioprotective agent to the patients with cardiovascular diseases.

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The authors declare that they have no conflict of interest. This work was supported by Mashhad University of Medical Sciences, IRAN.

**REFERENCES**


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Fright from snakes is as primitive as human civilization and perhaps it is the instinct of human. Buddhists and Hindus regarded them sacred as they appear in myths and tales while other abhorred them because of threatening to the lives. It is alarming that the most devastating rural community issue in tropical and subtropical areas of the world is snake bite envenomation. South Asia is by far the most affected region due to a lot of inhabitants, abundance of snake species, agricultural activities and ignorance about snake bite control programs. However, open style habitation and habit of laying on the floor increases the risk of snake bite (1). In Pakistan, eighty different species of snakes are found but fifteen are deadly poisonous. They belong to elapidae, viperidae and hydrophiidae families. Genus Naja (being elapidae) is quite ubiquitous and constitute ten species of Asiatic Naja. Naja naja karachiensis is one of them and mostly victimize people of southern Punjab province of Pakistan (2, 3). It has been estimated that 20,000 bites occur in Pakistan, which result in 8,200 deaths annually (1, 4).

Naja naja karachiensis envenomation produced severe complications in sufferers like hypotension, edema, pain, paralysis, necrosis, cardiac arrest, mucus discharge, bleeding gums, bleeding wounds, hematuria and anticoagulation (4-6).

Abstract: Present study was carried out regarding enzymatic assay for 5'-nucleotidase enzymes present in snake venom Naja naja karachiensis and to evaluate twenty eight medicinal plants as their antidotes. Elevated enzymatic activities i.e., 119, 183, 262 and 335 U/mL were observed in 10, 20, 30 and 40 µg of crude venom, respectively, in dose dependent manner. Among various plant extracts only two (Rauhina variegata L. and Citrus limon (L.) Burm. f.) were found 94% effective at 160 µg to neutralize 112 U/mL activities (p ≥ 0.5) while reference standard was proved 93.2% useful at 80 µg to halt 111 U/mL activities. Cedrus deodara G. Don, Enicostemma hyssopifolium (Willd.) Verdoorn, Terminalia arjuna Wight & Am. and Zingiber officinale Rosc. (at ≥ 160 µg) were found ≥ 90% effective (0.5 ≥ p ≥ 0.1) while Citrullus colocynthis, Fagonia cretica L., Rhazya stricta Dcne and Stenolobium stans (L.) D. Don (at ≥ 320 µg) were proved ≥ 90% effective (0.05 ≥ p ≥ 0.02). The remaining plant extracts were observed abortive (p ≥ 0.001) in neutralization of 5'-nucleotidases enzymatic actions. This study emphasizes further characterization of active plant extracts to further explore the anti-venom influences of these herbal remedies against deleterious effects produced by 5'-nucleotidase enzymes after snake bite envenomation.

Keywords: 5'-nucleotidases, medicinal plants, Naja naja karachiensis, anti-sera
is mainly attributed to the phospholipase A2 (PLA₂) and protease enzymes although deleterious effect of 5’-nucleotidases cannot be ignored (7, 8). For the first time, Dhananjaya et al. highlighted tremendous role of 5’-nucleotidases as platelet aggregation inhibitor (9). Since then, they were considered to delay blood coagulation synergistically with other toxins like PLA₂, ADPases and disintegrins. Being ubiquitous and glycoprotein in nature they were evaluated previously for neutralization by various inhibitors. However, majorities were unable to nullify them due to complex nature of protein and lack in specificity (9).

Specific inhibitors of 5’-nucleotidases are pharmacological and biochemical tools to characterize these enzymes (9). Natural antidotes, particularly medicinal plants have been documented previously to neutralize various snakes’ venom enzymes (10). The literature study reveals anti-venom activity of various plants and their constituents as well as the elucidation of the mechanism of action of various toxins/enzymes (9, 11). Pakistan is gifted with a rich capital of medicinal plants where varieties of them have been used locally to treat snakebite (12). It is therefore, necessary to rationalize scientifically their ethnobotanical claim as anti-snake venom (anti-5’-nucleotidases). Due to

Table 1. Detail description about Pakistani medicinal plants used to inhibit snake venom 5’-nucleotidases.

<table>
<thead>
<tr>
<th>No.</th>
<th>Medicinal plant (Family)</th>
<th>Collection (Location, part selected)</th>
<th>Voucher No.</th>
<th>Ref. no. (anti-snake venom)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Albizia lebbeck (L.) Benth. (Mimosaceae)</td>
<td>(Bahawalpur, seed)</td>
<td>STW.381</td>
<td>18</td>
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<td>2</td>
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<td>(Bhakkar, bulb)</td>
<td>STW.42</td>
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<td>3</td>
<td>Allium sativum L. (Liliaceae)</td>
<td>(Bhakkar, bulb)</td>
<td>STW.46</td>
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<tr>
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<td>Althaea officinalis L. (Malvaceae)</td>
<td>(Rawalpindi, roots)</td>
<td>STW.411</td>
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<tr>
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<td>Bauhinia variegata L. (Caesalpiniaceae)</td>
<td>(Haripur, roots)</td>
<td>STW.374</td>
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<td>(Manshera, seeds)</td>
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<td>(Haripur, exudates and flowers)</td>
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<td>(Nathia Gali, bark)</td>
<td>STW.25</td>
<td>18</td>
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<td>(Haripur, fruit)</td>
<td>STW. XX</td>
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<td>(Bahawalpur, fruits)</td>
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<tr>
<td>11</td>
<td>Cuminum cyminum L. (Apiaceae)</td>
<td>(Sargodha, seeds)</td>
<td>STW.516</td>
<td>18</td>
</tr>
<tr>
<td>12</td>
<td>Enicostemma hyssopifolium (Willd.) Verdooom (Gentianaceae)</td>
<td>(Jhelum, full plant)</td>
<td>STW.553</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>Fagonia cretica L. (Zygophyllaceae)</td>
<td>(Lasbella, leaves)</td>
<td>STW.433</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>Leucas capitata Desf. (Lamiaceae)</td>
<td>(Rawalpindi, full plant)</td>
<td>STW.615</td>
<td>21</td>
</tr>
<tr>
<td>15</td>
<td>Matthiola incana (L.) R. Br. (Cruciferae)</td>
<td>(Rawalpindi, seeds)</td>
<td>STW.322</td>
<td>18</td>
</tr>
<tr>
<td>16</td>
<td>Momordica charantia L. (Cucurbitaceae)</td>
<td>(Abbottabad, fruit)</td>
<td>STW.706</td>
<td>18</td>
</tr>
<tr>
<td>17</td>
<td>Nerium indicum Mill. (Apocynaceae)</td>
<td>(Haripur, roots &amp; leaves)</td>
<td>STW.564</td>
<td>12</td>
</tr>
<tr>
<td>18</td>
<td>Ocimum sanctum (Lamiaceae)</td>
<td>(Islamabad, full plant)</td>
<td>STW.626</td>
<td>24</td>
</tr>
<tr>
<td>19</td>
<td>Pinus roxburghii Sargent (Pinaceae)</td>
<td>(Murree, oleoresin)</td>
<td>STW.26</td>
<td>18</td>
</tr>
<tr>
<td>20</td>
<td>Pistacia integerrima (Anacardiaceae)</td>
<td>(Murree, galls)</td>
<td>STW.458</td>
<td>18</td>
</tr>
<tr>
<td>21</td>
<td>Psoralea corylifolia L. (Papilionaceae)</td>
<td>(Peshawar, seeds)</td>
<td>STW.418</td>
<td>18</td>
</tr>
<tr>
<td>22</td>
<td>Rheuza stricta Dcne (Apocynaceae)</td>
<td>(Lakki Marwat, leaves )</td>
<td>STW.565</td>
<td>12</td>
</tr>
<tr>
<td>23</td>
<td>Rubia cordifolia (Rubiaceae)</td>
<td>(Murree, stems)</td>
<td>STW.689</td>
<td>18</td>
</tr>
<tr>
<td>24</td>
<td>Sapindus mukorossi Gaertn. (Sapindaceae)</td>
<td>(local market, fruits)</td>
<td>STW.463</td>
<td>25</td>
</tr>
<tr>
<td>25</td>
<td>Stenolobium stanis (L.) D. Don (Bignoniaceae)</td>
<td>(Haripur, roots)</td>
<td>STW.669</td>
<td>18</td>
</tr>
<tr>
<td>26</td>
<td>Terminalia arjuna Wight and Arn. (Combretaceae)</td>
<td>(Islamabad, bark)</td>
<td>STW.502</td>
<td>18, 24</td>
</tr>
<tr>
<td>27</td>
<td>Trichodesma indicum (L.) R. Br. (Boraginaceae)</td>
<td>(Sind, whole plant)</td>
<td>STW.604</td>
<td>18</td>
</tr>
<tr>
<td>28</td>
<td>Zingiber officinalis Rosc. (Zingiberaceae)</td>
<td>(Lahore, rhizome)</td>
<td>STW.66</td>
<td>26</td>
</tr>
</tbody>
</table>

**MATERIALS AND METHODS**

Collection of snakes

*Naja naja karachiensis* snakes were collected with the help of snake charmers from Cholistan desert located in southern Punjab province of Pakistan. They were collected after duly identification by zoologist.

Milking of snakes venom

Cobra venom was extracted from snakes by squeezing the glands below their eyes in low light environment. The venom was freeze dried and preserved in a light resistant bottle at 2-8°C. Further, it was used in terms of dry weight (13).

Collection of plants material

The reported medicinally effective parts of various plants were collected from different locations in Pakistan as listed in Table 1. After collection, they were properly authenticated by renowned botanist Prof. Dr. Altaf Ahmad Dasti. Voucher specimens were deposited in the herbarium of the Institute of Pure and Applied Biology, Bahauddin-Zakariya University, Multan, Pakistan.

Extraction of plants material

After washing and shade drying, different parts of the plants were chopped and 1 kg of each was soaked in 5 L of methanol. They were kept in extraction bottles for a period of one month. They were filtered initially by ordinary paper followed by filter paper no 41. Filtrate was evaporated in water bath to get crude plant extracts. Subsequently, they were weighed and preserved for further experimentation (14).

Standard antidote (anti-venom)

Reference standard anti-sera were purchased from local pharmacy of Nishtar Hospital Multan Pakistan. They were manufactured by Bharat Serums and Vaccines Limited, Ambernath (E) – 421 501, India (4).

Enzymatic assay for snake venom 5'-nucleotidases

Enzymatic assay for 5'-nucleotidases was performed by using 5'-AMP as a substrate. Briefly, venom solution (0.1 mL, 10-40 µg) was added to the reaction mixture containing 5'-AMP (0.5 mL, 0.02 M), glycine (0.5 mL, 0.2 M) and magnesium sulfate (0.1 mL, 0.2 M). Further, it was subjected to incubation at 37°C for a period of 10 min, then trichloroacetic acid (1.5 mL, 10%) was added to stop the reaction. Inorganic phosphate contents were determined by addition of ascorbic acid reagent. Reaction mixture was kept at room temperature for half an hour and absorbance was measured at 820 nm after subtracting from the value of sample blank (for detail of this experiment, reference (15) may be consulted). Reference curve with known concentration (µM) of inorganic phosphate was established and the results (units) are expressed in µmoles of phosphate liberated/min/mL (9).

For evaluation of anti-venom potentials, pre-incubated medicinal plant extracts (10-640 µg/0.1 mL) with snake venom (10 µg) were subjected to enzymatic assay for 15 min at 37°C (10). All experiments were performed four times for the sake of confirmation.

### Table 2. Dose dependend effect of 5'-nucleotidases enzymes present in snake venom *Naja naja karachiensis*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Dose of venom (µg/0.1 mL)</th>
<th>Absorbance (820 nm) of inorganic phosphate released (mean ± SEM) (n = 4)</th>
<th>5'-Nucleotidases activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>0.850 ± 0.184</td>
<td>119</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>1.303 ± 0.269</td>
<td>183</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>1.856 ± 0.274</td>
<td>262</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>2.369 ± 0.261</td>
<td>335</td>
</tr>
</tbody>
</table>
Statistics

All numerical values were mentioned as the mean ± SEM. They were calculated by Microsoft Excel 2007. Paired t test was applied under the guidelines and instructions published in British Medical Journal (16).

RESULTS AND DISCUSSION

Enzymatic role of 5′-nucleotidases was assessed by their hydrolytic actions on adenosine monophosphate (AMP). They resulted in release of inorganic phosphate, therefore standard curve for it was constructed as shown in Figure 1. A straight line was obtained with positive correlation constant \( r \geq 0.999 \), which confirmed complete correlation between two variables (absorbance vs. concentration). The presence of 5′-nucleotidases in cobra venom was determined by their successive elevated concentrations (10-40 µg) in liberation of inorganic phosphate. Little dose of venom was stuffed with less amount of enzyme and vice versa. Various enzymatic activities i.e., 119, 183, 262 and 335 U/mL were found in 10, 20, 30 and 40 µg of crude venom, respectively, as summarized in Table 2.

Venom was fixed at concentration of 10 µg/0.1 mL in the assay mixture to evaluate anti-venom potentials of various antidotes. Standard antidote at concentration of 80 µg was found to neutralize (111 U/mL) of 5′-nucleotidases activity. It was found 93.2% effect to switch off these enzymes. Upon comparison of various plant extracts with reference standard only two (Bauhinia variegate L. and Citrus limon (L.) Burm. f.) were found almost equally effec-

![Figure 1. Standard curve for inorganic phosphate released in terms of absorbance at 820 nm](image1.png)

![Figure 2. Maximum inhibitory potentials posed by the most effective plant extracts in comparison with reference standard (p > 0.5) to neutralize Naja naja karachiensis 5′-nucleotidases activity](image2.png)
5’-Nucleotidases of *Naja naja karachiensis* snake venom...

Table 3. Anti-venom potentials of various medicinal plant extracts (in comparison with standard antidote) upon *Naja naja karachiensis* venom induced 5’-nucleotidases toxicity.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name of antidote</th>
<th>Activity of 5’-nucleotidases inhibited in U/mL (at most effective concentration)</th>
<th>Activity of 5’-nucleotidases retained (U/mL)</th>
<th>Inhibition of 5’-nucleotidases activity (%)</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Albizia lebbeck (L.) Benth.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Allium cepa L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Allium sativum L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Althaea officinalis L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Bauhinia variegata L.</td>
<td>112 (160 µg) 7 94</td>
<td>p ≥ 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Brassica nigra (L. Koch)</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Calotropis procera (Wild.) R. Br. (exudates)</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Calotropis procera (Wild.) R. Br. (flower)</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Cedrus deodara G. Don</td>
<td>106.8 (160 µg) 12.2 90</td>
<td>p ≥ 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Citrullus colocynthis</td>
<td>114 (320 µg) 5 95.7</td>
<td>p ≥ 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Citrus limon (L.) Burm. f.</td>
<td>112 (160 µg) 7 94</td>
<td>p ≥ 0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Cuminum cyanimum L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Enicostemma hyssopifolium (Wild.) Verdoorn</td>
<td>109.5 (320 µg) 9.5 92</td>
<td>p ≥ 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Fogonia cretica L.</td>
<td>102.5 (320 µg) 16.5 86</td>
<td>p ≥ 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Leucas capitata Desf.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Matthiola incana (L.) R. Br.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Momordica charantia L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Nerium indicum Mill.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Ocimum sanctum</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Pinus roxburghii Sargent</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Pistacia integerrima</td>
<td>104 (640 µg) 15 87.3</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>Psoralia corylifolia L.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>Rhazya stricta Dcne</td>
<td>97.8 (640 µg) 21.2 82</td>
<td>p ≥ 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Rubia cordifolia</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Sapindus mukorossi Gaertn.</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>Stenolobium stans (L.) D. Don</td>
<td>96.7 (320 µg) 22.3 81</td>
<td>p ≥ 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>Terminalia arjuna Wight &amp; Arn.</td>
<td>113.2 (160 µg) 5.8 95</td>
<td>p ≥ 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>Trichodesma indicum L R.Br</td>
<td>Nil Total present 0</td>
<td>p ≥ 0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>29</td>
<td>Zingiber officinalis Rosc.</td>
<td>112.4 (320 µg) 6.6 94.4</td>
<td>p ≥ 0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Standard antidote (anti-sera)</td>
<td>111 (80 µg) 8 93.2</td>
<td>Select to compare</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

So far, some plant extracts were also proved ≥ 90% effective (0.5 ≥ p ≥ 0.1) at concentration of ≥ 160 µg. Among them Cedrus deodara G. Don, Enicostemma hyssopifolium (Wild.) Verdoorn, Terminalia arjuna Wight & Arn. and Zingiber officinalis Rosc. are included. *Citrullus colocynthis, Fogonia cretica L., Rhazya stricta Dcne and Stenolobium stans (L.) D. Don* were proved less effective (0.05 ≥ p ≥ 0.02) even at higher concentration (≥ 320 µg). The remaining plant extracts were proved abortive (p ≥ 0.001) in neutralization of 5’-nucleotidases present in snake venom *Naja naja karachiensis*. Overall details about anti-venom potentials of medicinal plants are summarized in Table 3.
5’-Nucleotidases are the most lethal components of the snake’s venom that impart miscellaneous toxicities upon severe poisoning. They occur in excess in different snake’s venom and have been documented previously like *Naja naja*, *Trimeresurus graminus* and *Vipera aspis* (17). Snake bite envenomation has been reported to break various substrate(s) in the victims, resulting in production of 5’-nucleotides (5’-AMP). However, adenosine (multi-toxin) moieties are subsequently generated by the enzymatic action of 5’-nucleotidases. Adenosine binds to various adenosine (A1 and A2) receptors in the body and promotes cardiac arrest, redness, inflammation, renal damage, cognitive impairment, neurotransmitter imbalance and antiplatelet aggregation, hence the delay occurs in blood coagulation. Moreover, it also binds to the A1A and A2B receptors further resulting in dilation of blood vessels, which fortified the concept of coagulopathies produced by *Naja naja karachiensis* envenomation (17). Due to these detrimental effects in victims, present study was conducted to confirm the presence of 5’-nucleotidases in Pakistani cobra venom so that possible toxicities owing to these enzymes could be eradicated.

Natural antidotes have gained therapeutic relevance to neutralize various snake venom enzymes. Medicinal plants have earliest history to neutralize various enzymes due to the abundance of secondary metabolites. Phenols, quinonoids, xanthenes, terpenoids and flavonoids were reported previously to inhibit various poisonous proteins of snake’s venom (12). Secondary metabolites in various plant extracts (especially *Bauhinia variegate* L. and *Citrus limon* (L.) Burm. f.) created disturbances in binding of 5’-nucleotidases to their receptor(s), therefore resulted in recovery of different toxicities.

**CONCLUSION**

Conclusively, *Bauhinia variegate* L. and *Citrus limon* (L.) Burm. f. have been found as useful as standard antidote (reference standard) to neutralize 5’-nucleotidases. These natural herbs are potential cheap alternate therapies to overcome the complications posed by phospholipases (PLA), proteases, and L-amino oxidases.

**Conflict of interest**

Authors have found no competing interests.

**Acknowledgment**

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**REFERENCES**


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Sulfur, selenium, and tellurium are in the same group - VIA in the Periodic Table. It has been reported that sulfur is less toxic than selenium (1). Sodium tellurite (Te) has applications in various industries; steel, rubber, electronic, ceramic and glass. Te demonstrates properties similar to those of elements known to be toxic to humans and animals (2). The organic tellurium compound, diphenyl ditelluride at the dose of 0.65 µmol/kg in rats has increased 2-fold serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities (3). The other organo tellurides, 2-butyltellurium furan and dinaphthalene ditelluride at a dose of 0.75 and 0.125 mmol/kg have also increased the activities of AST and ALT in the liver of rats (4). Conversely, the vinyl telluride derivative, (diethyl-2-phenyl-2-tellurophenylvinyl-phosphonate) has protected the hepatotoxicity in rodents (5). It has also protected the levels of lipid peroxidation (5). Similarly, the other vinyl tellurium compounds, [(Z)-2-(methylthio)-1-(butyltelluro)-1-phenylethene] have shown antioxidant properties in vitro and in vivo (6). Te accelerates the toxicity of Cd, so it is used as cadmium telluride quantum dots (CdTe-QDs), which accelerates the formation of lipid peroxidation and decreased the activities of catalase (CAT) and superoxide dismutase (SOD) in the liver of mice (7). CdTe-QDs caused cytotoxicity in HepG2 cells and increased the reactive oxygen species (ROS), decreased the level of glutathione (GSH) and activities of CAT and glutathione-S-transferase (GST) (8).

Kaur et al., (9) have reported 2-fold more neurotoxicity of Te than Se on the same parameters.
Sodium tellurite has been reported to cause neurotoxicity by decreasing the contents of the lipids profiles in cerebrum, cerebellum and brainstem of mice (9). It has also depleted the content GSH and activities of antioxidant enzymes (GPx, GR, SOD, GST and CAT in the cerebrum, cerebellum and brainstem of mice (9). On the other hand, Te has elevated the level of lipid peroxidation in discrete brain areas of the animals (9). Moreover, Te causes toxicity by acting directly as a general oxidizing agent.

The above studies of organo-tellurium compounds or CdTe-QDs are available in the literature but the hepatotoxicity caused by inorganic Te compounds is not available which has created our interest to evaluate the devastating toxic effect of Te on the liver of the rats.

Te has been reported to bind with sulfhydryl components of squalene monooxygenase, thereby causing blockage in the formation of cholesterol, which could lead to peripheral neuropathy with significant demyelination with the passage of time (10-13). The depleted level of sulfhydryl group makes the cells more susceptible to free radicals.

**MATERIALS AND METHODS**

**Chemicals**

Sodium tellurite, oxidized glutathione (GSSG), reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), 1-chloro-2,4-dinitrobenzene (CDNB), ethylene diamine tetraacetic acid (EDTA), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), thiobarbituric acid (TBA), (-)-epinephrine, sodium azide, hydrogen peroxide, sulfosalicylic acid and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich, Co., Germany. The kits were purchased from Human Gesellschaft fur Biochemical and Diagnostic mbH, Germany.

**Animals and dosing**

The male Wistar rats 200-220 g were taken from the Animal House of Jazan University, Jazan, Kingdom of Saudi Arabia. All procedures were performed in accordance with NIH guidelines and Guide for the Care and Use of Animals. These protocols were approved by Jazan University Institutional Animal Care and Use Committee (IACUC). The animals were divided into four groups each having 8 animals. Group 1 was control group and vehicle (saline) was given orally. Groups 2-4 were experimental and sodium telluride 4.15, 8.3 and 16.6 mg/kg body weight (b.w.) 1/20, 1/10 and 1/5 of LD50, respectively; oral LD50 in rat is 83 mg/kg) were given orally in saline for 15 days. On day 16, blood was taken out from orbital sinus of overnight fasted animals from each group for biochemical assays. Thereafter, animals were sacrificed and liver of each animal was dissected out. A 10% homogenate of the liver was prepared in 20 mM Tris-HCl (pH 7.4, having protease inhibitors 10 µl/mL). The homogenate was centrifuged at 800 × g for 5 min at 4°C to remove cell debris. The supernatant-1 (S-1) was used for lipid peroxidation and rest of the S-1 was again centrifuged at 10,500 × g for 30 min at 4°C to separate the post mitochondrial supernatant (PMS). The PMS was used for the assays of GSH, GPx, GR, GST, SOD and CAT.

**Biochemical assays for liver function tests**

The kits of Human Gesellschaft fur Biochemical and Diagnostic were used for the assays of all serum markers such as bilirubin, AST, ALT and ALP.

**Non enzymatic assays**

**Lipid peroxidation (LPO)**

The procedure of Utley et al. (14) as modified by Islam et al. (15) was used for the estimation of the rate of lipid peroxidation. In brief, S-1 0.5 mL samples were pipetted in 18 × 150 mm test tubes and incubated at 37 ±1°C in a metabolic shaker (120 cycles / min) for 1 h. Another 0.5 mL of the same homogenate was pipetted in other test tube and placed at 0°C. After 1 h of incubation, 0.5 mL of chilled trichloacetic acid (TCA) followed by 1.0 mL of 0.67% thiobarbituric acid (TBA) were added to each test tube and mixed after each addition. The aliquot of each test tube was transferred to centrifuge tubes and centrifuged at 1000 ◊ g for 10 min at 4°C. Thereafter, supernatants were transferred to other test tubes and placed in the boiling water bath. After 10 min, the test tubes were cooled and the absorbance of the color was read at 535 nm. The rate of thiobarbituric acid reactive substances (TBARS) formed was expressed as nmol of TBARS formed/h/mg protein using molar extinction coefficient of 1.56 × 105 M-1 cm-1.

**Glutathione reduced (GSH)**

The method of Jollow et al. (16) was used for the assay of GSH. PMS 0.1 mL was precipitated with 0.1 mL of sulfosalicylic acid (4%). The samples were kept at 4°C for 1 h and then subjected to centrifugation at 1000 × g for 10 min at 4°C. The assay mixture contained 0.1 mL supernatant, 0.8 mL phosphate buffer (0.1 M, pH 7.4) and 0.1 mL DTNB (0.4% in phosphate buffer 0.1 M, pH 7.4) in a total
Repeated exposure of sodium tellurite on the rat liver and on the potential...

volume of 1.0 mL. The yellow color developed was read immediately at 412 nm. The GSH content was calculated as µmol GSH/mg protein, using molar extinction coefficient of 13.6 × 10^3 M⁻¹ cm⁻¹.

Assays of antioxidant enzymes

**Glutathione peroxidase (GPx)**

The method of Mohandas et al. (17) was used for the assay of GPx. In brief, the reaction mixture consisted of phosphate buffer (0.1 M, pH 7.0), 0.1 mL of 20 mM EDTA, 0.1 mL of 20 mM sodium azide, glutathione reductase (1U/mL), 0.1 mL of 20 mM glutathione, 0.1 mL of 4 mM NADPH, 0.1 mL of 5 mM of hydrogen peroxide and 0.1 mL of PMS in a total volume of 2 mL. The disappearance of NADPH at 340 nm was recorded per min for 3 min at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein using molar extinction coefficient of 6.22 × 10⁻³ M⁻¹ cm⁻¹.

**Glutathione reductase (GR)**

GR was assayed by the method of Carlberg and Mannervik (18) as modified by Mohandas et al. (17). In brief, the assay mixture consisted of phosphate buffer (0.1 M, pH 7.6), 0.1 mL of 2 mM NADPH, 0.1 mL of 10 mM EDTA, 0.1 mL of 20 mM oxidized glutathione and 0.1 mL of PMS to give a total volume of 3 mL. Change in absorbance was recorded at 240 nm per min for 3 min. The catalase activity was calculated in terms of nmols of H₂O₂ consumed/min/mg protein by using molar extinction coefficient of 43.6 M⁻¹ cm⁻¹.

**Glutathione-S-transferase (GST)**

The activity of GST was measured by the method of Habig et al. (19). The reaction mixture consisted of phosphate buffer (0.1 M, pH 6.5), 0.1 mL of 20 mM of reduced glutathione, 0.1 mL of 20 mM of 1-chloro-2,4-dinitrobenzene (CDNB) and 0.1 mL of PMS in a total volume of 2.0 mL. The change in absorbance was recorded at 340 nm per min for 3 min and enzyme activity was calculated as nmols CDNB conjugate formed/min/mg protein using molar extinction coefficient 9.6 × 10⁻³ M⁻¹ cm⁻¹.

**Catalase (CAT)**

The activity of CAT was measured by the method of Claiborne (20). In brief, the assay mixture consisted of phosphate buffer (0.1 M, pH 7.4), 0.1 mL of 180 mM of hydrogen peroxide and 0.05 mL PMS to give a total volume of 3.0 mL. Change in absorbance was recorded at 240 nm per min for 3 min. The catalase activity was calculated in terms of nmols of H₂O₂ consumed/min/mg protein by using molar extinction coefficient of 4.02 ×10³ M⁻¹ cm⁻¹.

**Superoxide dismutase (SOD)**

SOD activity was measured spectrophotometrically as described previously by Stevens et al. (21) by monitoring the autooxidation of (-)-epinephrine at pH 10.4 for 3 min at 480 nm. The reaction mixture contained glycine buffer (50 mM, pH, 10.4) and 0.2 mL of PMS. The reaction was initiated by adding 0.05 mL of 20 mM (-)-epinephrine. The enzyme activity per min for 3 min was calculated in terms of nmol (-)-epinephrine protected from oxidation/min/mg protein using molar extinction coefficient of 4.02 ×10³ M⁻¹ cm⁻¹.

**Estimation of protein**

Protein was estimated according to the method of Lowry et al. (22) using bovine serum albumin as standard.

### Table 1. Effects of sodium tellurite on bilirubin, AST, ALT and ALP in the serum of rats.

<table>
<thead>
<tr>
<th>Biomarker enzymes (Unit)</th>
<th>Control</th>
<th>Te 4.15 mg/kg</th>
<th>Te 8.3 mg/kg</th>
<th>Te 16.6 mg/kg</th>
<th>²F² value (MS treatment/MS residual)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilirubin (mg/dL)</td>
<td>0.34 ± 0.01</td>
<td>0.66 * ± 0.03 (92.42 %)</td>
<td>1.12 ** ± 0.06 (226.53 %)</td>
<td>1.5 ** ± 0.06 (291.54 %)</td>
<td>120.83</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>18.00 ± 0.23</td>
<td>61.60 ** ± 0.64 (242.22 %)</td>
<td>69.76 ** ± 0.96 (287.55 %)</td>
<td>70.54 ** ± 1.86 (291.89 %)</td>
<td>510.30</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>6.6 ± 0.31</td>
<td>18.2 ** ± 0.28 (175.75 %)</td>
<td>21.34 ** ± 0.45 (223.33 %)</td>
<td>25.18 ** ± 0.53 (281.51 %)</td>
<td>390.86</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>515.47 ± 0.68</td>
<td>1290.9 ** ± 46.31 (150.44 %)</td>
<td>1309.67 ** ± 45.16 (154.08 %)</td>
<td>1380.87 ** ± 50.28 (167.89 %)</td>
<td>99.02</td>
</tr>
</tbody>
</table>

Note: Te toxicity has significantly elevated the hepatotoxicity biomarkers in the serum of rats. Values are expressed as the mean ± S.E.M. of 8 rat/group. Values in parentheses show the percentage increase with respect to control.*p < 0.05, **p < 0.001 vs. control.
Statistics
The one-way ANOVA and post hoc Dunnett’s test were used for the significance and \( p < 0.05 \) was considered as significant.

RESULTS AND DISCUSSION
Table 1 shows the effects of sodium tellurite on the biomarkers of liver function tests (bilirubin, AST, ALT and ALP). The content of bilirubin was increased significantly \((^* p < 0.05, ^{**} p < 0.001)\) and dose dependently in the serum of the rats treated with various doses of Te as compared to control group. The rise in bilirubin level is one of the most important clinical indications of the severity of necrosis, and its accumulation further indicates the binding, conjugation and excretory capacity of hepatic cells (23). The significantly elevated level of bilirubin in the serum indicates that Te is hepatotoxic. No other data of inorganic or organic Te compounds on the content of bilirubin is available.

AST and ALT are the most sensitive biomarkers of liver toxicity that can directly indicate the extent of hepatic damage and toxicity. Administration of Te to rats caused significant elevation \((^* p < 0.001)\) on the activities of AST and ALT in the serum, indicating hepatotoxicity of Te. Elevation of ALP may be found in a large number of disorders such as gallstone disease, alcohol abuse and drug-induced hepatitis, or in less common disorders such as primary biliary cirrhosis or biliary tumors (24, 25). There is no report of inorganic tellurium compounds on the toxicity of liver biomarkers but organo tellurides: 2-butyltellurium furan, dinaphtha-
lone ditelluride and diphenyl ditelluride have significantly increased the activities of AST and ALT in the serum of rats (26). In the present study, the activity of alkaline phosphatase was increased significantly (**p < 0.001) and dose dependently in the serum of rats. The increased activity of ALP was supported by Srivastava et al. (27) in liver and kidney with the treatment of 2-butyltellurium furan and dinaphthalene ditelluride.

Figure 1 shows the effect of various doses of sodium tellurite on the contents of lipid peroxidation in the liver of rats. The level of TBARS was increased significantly (**p < 0.001) and dose dependently in the serum of rats. The increased activity of ALP was supported by Srivastava et al. (27) in liver and kidney with the treatment of 2-butyltellurium furan and dinaphthalene ditelluride.

Figure 3. Effect of various doses of sodium tellurite on activities of glutathione peroxidase (GPx) and glutathione reductase (GR) in the liver of rats. The activities of GPx and GR were decreased significantly and dose dependently in sodium tellurite (4.15, 8.3 and 16.6 mg/kg) treated groups as compared to control group. The values are expressed as the mean ± S.E.M (n = 8 rats/group). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control.

Figure 4. Effect of various doses of sodium tellurite on the activity of glutathione-S-transferase (GST) in the liver of rats. The activity of GST was decreased significantly and dose dependently in sodium tellurite (4.15, 8.3 and 16.6 mg/kg) treated groups as compared to control group. The values are expressed as the mean ± S.E.M (n = 8 rats/group). *p < 0.01 and **p < 0.001 vs. control.

Vinyl telluride derivative - (Z)-1-(4-methylphenylsulfonyl)-2-(phenyltelluro)-2-phenylethene has increased hepatic lipid peroxidation in rats. Conversely, other vinyl telluride derivative; (Z)-2-(methylthio)-1-(butyltelluro)-1-phenylethene has reducing effect on lipid peroxidation in vitro. At very low concentration, the CdTe-QDs (500 nmole/mL) has increased the TBARS concentration in the liver tissue (7). A supporting data of sodium tellurite toxicity on TBARS in the brain of mice have been reported (9).

The content of GSH was decreased significantly (*p < 0.05 and **p < 0.001) and dose dependently in Te treated groups as compared to control group (Fig. 2). GSH also plays a crucial role in regulation of the expression of several redox-sensitive antioxidant and anti-inflammatory genes (28, 29). Thus, GSH inhibition in the liver has increased the suscep-
tibility of plasma membranes towards peroxide attacks. The loss of GSH and formation of protein–glutathione mixed disulfide (PrSSG) may result in various membrane dysfunctions, such as inhibition of Na⁺K⁺-ATPase activity (30). The significantly depleted content of GSH has also been reported in the liver and kidney of rats treated with organo-tellurium compound, bis-(tetraphenylphosphonium) tetracyanato-bis-p-methoxy-p-phenyl tellurate and bis-(tetraheptylammonium) tetraiodocyclopentane tellurate (27). Sodium telluride has also depleted the contents of GSH in the brain of mice (9).

Reactive free radicals such as superoxide and hydroxyl can damage lipid, protein, or DNA and cause cell death (8). Se and Te have been known for some time as thiol-reactive reagents, although the nature of the chemistry is still not completely understood (7). The generation of overproduction of free radicals, which might have caused oxidative damage to the membrane lipid and protein, ultimately led to a decrease in GSH content and activity of antioxidant enzymes. The activity of NADPH dependent antioxidant enzymes; GPx and GR was decreased significantly (*p < 0.05, **p < 0.01 and ***p < 0.001) and dose-dependently in the PMS of the liver of Te treated groups as compared to control group (Fig. 3). GPx plays a predominant role in removing the excess free radicals and hydroperoxides and is a major defense system against oxidative stress (31). GR also plays a significant role in catalyzing the oxidized glutathione (GSSG) to GSH. During the normal catalytic reaction of GPx and GR, the consumed glutathione is recycled. The decreased activities of GPx and GR were also reported in the brain of the mice with the treatment of sodium telluride (9).

During the generation of glutathione-S-conjugates by GST, the GSH is consumed by the cells due to which the total intracellular GSH contents are...

Figure 5. Effect of various doses of sodium tellurite on the activity of catalase (CAT) in the liver tissue of rats. The Te intoxication has decreased the activity of CAT significantly and dose dependently (4.15, 8.3 and 16.6 mg/kg) in the liver as compared to control group. The values are expressed as the mean ± MSE. (n = 8 rat/group). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control

Figure 6. Effect of various doses of sodium tellurite on the activity of superoxide dismutase (SOD) in the liver tissue of rats. The Te intoxication has decreased the activity of SOD significantly and dose dependently (4.15, 8.3 and 16.6 mg/kg) in the liver as compared to control group. The values are expressed as the mean ± S.E.M (n = 8 rat/group). *p < 0.01 and **p < 0.001 vs. control
lowered which caused more harm to the cells. The activity of GST was decreased significantly (*p < 0.01 and **p < 0.001) and dose dependently in the PMS of the liver of Te treated groups as compared to control group (Fig. 4). The decreased activity of GST was supported by the treatment of sodium tellurite in the brain of mice as well as organo-tellurium compounds; bis-(tetraphenylphosphonium) tetracyanato-bis-p-methoxy-p-phenyl tellurate and bis-(tetraheptylammonium) tetraiodocyclopentane tellurate in liver and kidney of mice (9, 27).

The activity of other antioxidant enzymes such as SOD (*p < 0.01 and **p < 0.01) and CAT (*p < 0.05 and **p < 0.01 and ***p <0.001) were also decreased significantly and dose dependently in Te treated groups as compared to control group (Figs. 5 and 6). SOD converts highly toxic superoxide into less toxic H$_2$O$_2$ (32) and CAT detoxifies H$_2$O$_2$ into H$_2$O and oxygen. It has been reported that defense against H$_2$O$_2$, which is the most toxic molecule in the body, is mediated primarily by the glutathione system. The Te has depleted the activities of these enzymes due to which the level of H$_2$O$_2$ was not detoxified and caused liver toxicity. Kaur et al. (9) have reported the decreased activities of these enzymes in the discrete brain areas of mice. No other data of the Te (inorganic or organic) on the liver of SOD and CAT are available. So this is our first report on the toxicity of sodium tellurite on the activities of SOD and CAT in the liver of rats.

**CONCLUSION**

The toxicity of Te on hepatic biomarkers indicates that Te caused hepatotoxicity and generates free radicals which cause oxidative stress and more severity to the liver.

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**REFERENCES**


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Synthetic derivatives of indolo[2,3-b]quinolines constitute a novel group of compounds with a potent antitumor activity. The structure of indolo[2,3-b]quinolines is derived from neocryptolepine, a plant alkaloid isolated from the roots of the African shrub Cryptolepis sanguinolenta. Aqueous extracts of this plant (composed of the mixture of isomeric indoloquinolines) have been used for years in folk medicine as antimicrobial and antiplasmodial agents (1). A synthetic analog of neocryptolepine i.e., 5,11-dimethyl-5H-indolo[2,3-b]quinoline (DIMIQ), structurally similar to ellipticine – a plant alkaloid with a cytotoxic activity (2), was synthesized by our group in 1988 (3). It was found that the DIMIQ displayed a cytotoxic activity in vitro and in vivo against mouse leukemia P388, L1210 and B16 melanoma cell lines (3). However, more advanced research revealed impaired bioavailability of 5H-indolo[2,3-b]quinoline derivatives, which necessitated further studies on the modification of the indoloquinoline ring structure (4). Research on the structure-cytotoxic activity relationship of indolo[2,3-b]quinoline from series 5H- and 6H- revealed that the presence, position and nature of the substituent is crucial for the activity of these derivatives. Since 6,11-dimethyl-6H-indolo[2,3-b]quinoline is an inactive isomer of 5,11-dimethyl-5H-indolo[2,3-b]-quinoline (DIMIQ), it can acquire this activity following the introduction of an additional methyl...
group at 5 position (5) or an alkylaminoalkyl substituent (6, 7). Further structure-activity relationship analysis led us to the concept that carbohydrate indolo[2,3-b]quinoline hybrids could create a distinct group of compounds with a potential cytotoxic activity (8, 9). By analogy with the anthracycline antibiotics, a proper aminoglycosyl substituent can influence the ability of the whole molecule to form specific complexes with the DNA and topoisomerase II (10, 11). Moreover, a positive influence of the carbohydrate indolo[2,3-b]quinoline O-aminoglycosides was synthesized and evaluated in vitro for their cytotoxic activity against several cell lines of different origin. All the compounds tested show a cytotoxic activity against A549, MCF-7 and Hs294T cells and overcome multidrug resistance in colorectal adenocarcinoma LoVo/DX, uterine sarcoma MES-SA/DX5 and promyelocytic leukemia HL-60/MX2. These promising results obtained for the hybrids composed of a previously inactive 6H-indolo[2,3-b]quinoline and aminosaccharide moiety prompted us to synthesize and examine O-aminoglycosides of the active 5H-isomeric chromophore.

EXPERIMENTAL

Chemistry
Melting points (m.p.’s) were determined on a Kofler-type apparatus and are uncorrected. The IR spectra were recorded on Nicolet FT-IR Impact 410 and Beckman 4220 spectrophotometers in KBr pellets. The NMR spectra were recorded on Bruker AM-500 and Varian GEM 200 spectrometers; the chemical shifts are expressed in ppm (δ) with TMS as the internal standard. The MS spectra were recorded on an Intectra AMD-604 apparatus (EI, 70 eV). Elemental analyses were performed by the analytical laboratory of the Institute of Organic Chemistry of the Polish Academy of Sciences. The purity and identity of products were checked by thin layer chromatography (TLC) with Merck DC-Alufolien Kieselgel 60 F254. For column “flash” chromatography Kieselgel 60, 230-400 mesh (Merck) was used. Chemicals and solvents were purchased from Aldrich Company. Organic extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure.

5,11-Dimethyl-5H-indolo[2,3-b]quinolinol (3, 4) 

General procedure
The corresponding 2- or 9-methoxy-11-methyl-6H-indolo[2,3-b]quinoline 1 or 2 (10.0 g, 38 mmol) was suspended in toluene (150 mL), dimethyl sulfate (10 mL) was added and the whole mixture was stirred under reflux for 24 h. After cooling, the mixture was diluted with water, basified with 25% aq. ammonia and extracted with chloroform. The extract was diluted with water, evaporated in vacuo, the residue mixed with the solution of HBr in the acetic acid (33%, 150 mL) and refluxed for 24 h (TLC control). Then, the next portion of HBr in the acetic acid (50 mL) was added and the reflux continued for the next 8 h. The reaction mixture was cooled, diluted with water (200 mL), alkalized with 25% aq. ammonia and extracted with v/v 2% methanol-chloroform. The extract was dried over MgSO4, evaporated in vacuo and the residue purified by column chromatography, eluting with 2-20%, v/v methanol-chloroform.

5,11-Dimethyl-5H-indolo[2,3-b]quinolin-2-ol (3) 
Compound 1 was used as the starting material to give orange crystals, yield 897 mg (10%), m.p. above 280°C (decomp.).

IR (KBr, cm-1): 2933, 1632, 1609, 1578, 1492, 1443, 1351, 1332, 1241, 1138, 738; 1H NMR (DMSO-d6, δ ppm): 7.98 (s, 1H), 8.19 (d, 1H, J = 8 Hz), 7.84 (d, 1H, J = 9 Hz), 7.59-7.34 (m, 4H), 7.14 (t-d, 1H, J = 8 - 1 Hz), 4.24 (s, 3H), 3.03 (s, 3H); MS (m/e, %): 262 (M+1, 100), 247 (38); Analysis: for C17H14N2O ◊ 0.5 H2O (271.33) calcd.: 75.25% C, 5.57% H, 10.33% N; found: 75.20% C, 5.38% H, 10.15% N.

5,11-Dimethyl-5H-indolo[2,3-b]quinolin-9-ol (4) 

Compound 2 was used as the starting material to afford deep red crystals, yield 1148 mg (21%), m.p. above 280°C (decomp.).

IR (KBr, cm-1): 2924, 1626, 1609, 1572, 1528, 1496, 1452, 1283, 1131, 816, 745; 1H NMR (DMSO-d6, δ ppm): 8.98 (s, 1H), 8.28 (d-d, 1H, J = 8 - 1 Hz), 7.91 (d-d, 1H, J = 9 - 1 Hz), 7.82 (d-d-d, 1H, J = 8 - 7 - 1.5 Hz), 7.61 (d, 1H, J = 2 Hz), 7.48 (d-d-d, 1H, J = 8 - 6.5 - 1 Hz), 7.38 (d, 1H, J = 8.5 Hz), 6.95 (d-d, 1H, J = 8.5 - 1.5 Hz), 4.22 (s, 3H), 3.08 (s, 3H); MS (m/e, %): 262 (M+1, 100), 247 (44); Analysis: for C17H14N2O ◊ 0.5 H2O (271.32) calcd.: 75.25% C, 5.57% H, 10.33% N; found: 75.20% C, 5.38% H, 10.15% N.

(2S, 3R, 4S)-3-Acetoxy-2-methyl-1-trifluoracetamido-3,4-dihydro-2H-pyran (4-O-acetyl-3-N-trifluoracetetyl-L-ascosaminal) (6) and (2S, 3S, 4S)-2-methyl-3-(4-nitrobenzoyloxy)-4-trifluoracetamido-3,4-dihydro-2H-pyran (4-O-p-nitrobenzoyl-3-N-trifluoroacetyl-L-daunosaminal) (8)
General procedure
Tetrakis(triphenylphosphine)palladium (3.5 g) and formic acid (24 mL) were added to the solution of (2S, 3R, 4S)-3-acetoxy-4-allyloxycarbonyl-amino-2-methyl-3,4-dihydro-2H-pyran (5) (12) (6.0 g, 23.5 mmol) or (2S, 3S, 4S)-4-allyloxycarbonyl-amino-2-methyl-3-(4-nitrobenzoyloxy)-3,4-dihydro-2H-pyran (7) (12) (6.0 g, 16.5 mmol) in THF (250 mL) under inert atmosphere. The mixture was stirred at ambient temperature for 12 h, triethylamine (16 mL) and trifluoracetic acid were dropped in at 0°C and the whole mixture was stirred again at ambient temperature for 48 h. Then, the mixture was poured into saturated aq. ammonium chloride, stirred for 15 min. and extracted with chloroform. The extract was washed with water, dried over MgSO4 and evaporated in vacuo.

(2S, 3R, 4S)-3-Acetoxy-2-methyl-4-trifluoracetamido-tetrahydropyran (4-O-acetyl-3-N-trifluoracetyl-L-acosaminal) (6)
Compound 5 was used as the starting material yielding white crystals, yield 2.39 g (38%), m.p. 154-158°C.

IR (KBr, cm-1): 3290, 3103, 1746, 1656, 1559, 1381, 1238, 1185, 1060, 907, 728; 1H NMR (CDCl3, δ, ppm): 8.31-8.24 (m, 2H), 7.98-7.91 (m, 2H), 6.77 (bd, 1H, J = 8.5 Hz), 6.48 (d-d, 1H, J = 6.5 - 2 Hz), 4.97-4.91 (m, 1H), 4.68 (d-d, 1H, J = 6.5 - 2 Hz), 4.13 (bd, 1H, J = 6.5 Hz), 3.83 (m, 3H), 1.38 (d, 3H, J = 6.5 Hz); MS (m/e, %): 224 (M+ - Ac, 0.86), 206 (M+ - F, 4.55), 192 (100); Analysis: for C10H12NO4F3 (267.20) calcd.: 44.95% C, 4.53% H, 5.24% N; found: 45.03% C, 4.73% H, 5.16% N.

(2S, 3S, 4S)-2-Methyl-3-(4-nitrobenzoyloxy)-4-trifluoracetamido-3,4-dihydro-2H-pyran (4-O-p-nitrobenzoyl-3-N-trifluoracetyl-L-daunosaminal) (8)
Compound 7 was used as the starting material to afford pale yellow crystals, yield 1.06 g (17%), m.p. 140-143°C.

IR (KBr, cm-1): 3393, 3287, 3072, 2978, 2921, 1740, 1669, 1648, 1628, 1602, 1550, 1521, 1342, 1242, 1167, 1108, 1073, 989, 856; 1H NMR (CDCl3, δ, ppm): 8.31-8.24 (m, 2H), 7.98-7.91 (m, 2H), 6.77 (bd, 1H, J = 8.5 Hz), 6.48 (d-d, 1H, J = 6.5 - 2 Hz), 4.97-4.91 (m, 1H), 4.68 (d-d, 1H, J = 6.5 - 2 Hz), 4.13 (bd, 1H, J = 6.5 Hz), 3.83 (m, 3H), 1.38 (d, 3H, J = 6.5 Hz); MS (m/e, %): 261 (M+-NHTFA - H2), 260 (13), 245 (100), 150 (95); Analysis: for C11H11N2O6F3 x 2 Et2N (576.66) calcd.: 56.24% C, 7.52% H, 9.71% N; found: 56.34% C, 8.98% H, 9.24% N.

Sodium hydride (60% in oil) (240 mg, 6 mmol) was introduced to the solution of corresponding
indoloquinolinol 3 or 4 (786 mg, 3 mmol) in dry DFM (40 mL) at 0°C with stirring. After 30 min., the solution of the corresponding bromoalkylglycoside 9 or 10 (3 mmol) in dry DFM (20 mL) was added. The whole mixture was stirred at 0°C for 15 min. and for another 2 h at ambient temperature. Then, the reaction mixture was poured into saturated aqueous ammonia chloride (250 mL), stirred for 30 min. and extracted with 2% methanol in chloroform. The extract was then washed with water (50 mL), saturated aqueous NaHCO3 (50 mL), dried over magnesium sulfate and evaporated in vacuo. The residue was dissolved in chloroform-methanol 1:1, v/v (40 mL), potassium carbonate (500 mg) was added and the whole mixture was stirred at ambient temperature for 48–96 h. (TLC control). Then, the reaction mixture was poured into saturated aqueous NaHCO3 (50 mL), dried over magnesium sulfate and evaporated in vacuo. Then, the whole mixture was stirred at ambient temperature for 48–96 h. (TLC control). Then, the reaction mixture was poured into saturated aqueous NaHCO3 (50 mL), dried over magnesium sulfate and evaporated in vacuo. The crude product was purified by column chromatography using chloroform-methanol-triethylamine 80:20:0.06 and 60:20:0.05 v/v as the eluent.

The synthesis was performed using compounds 3 and 9, to give 11 as orange crystals, yield 654 mg (50%); m.p. 80–85°C.

IR (KBr, cm⁻¹): 3350, 2927, 1635, 1567, 1527, 1494, 1456, 1335, 1280, 1239, 1225, 1128, 1058, 981, 758; 'H NMR (DMSO-d₆, δ, ppm): 8.20 (d, 1H, J = 7.5 Hz), 7.89 (d, 1H, J = 9 Hz), 7.68 (d, 1H, J = 2.5 Hz), 7.57-7.40 (m, 3H), 7.25-7.11 (m, 2H), 4.41 (m, 1H), 4.29 (s, 3H), 4.18 (ABq, 2H), 3.79-3.48 (m, 2H), 3.13 (s, 3H), 2.98-2.80 (m, 1H), 1.90-1.74 (m, 2H), 1.70-1.44 (m, 5H), 1.17 (d, 3H, J = 6.5 Hz); MS (m/e, %): 477 (M⁺, 41), 377 (100); Analysis: for C₂₅H₂₉N₃O₄ (489.55) calcd.: 61.69% C, 7.21% H, 8.58% N; found: 61.69% C, 7.07% H, 8.23% N.

2-[5-(α-L-Daunosaminloxy)penthox]-5,11-dimethyl-5H-indolo[2,3-b]quinoline (13)

The synthesis was performed using compounds 3 and 10, to give 13 as orange crystals, yield 476 mg (35%); m.p. 115–120°C.

IR (KBr, cm⁻¹): 3367, 3201, 2930, 1634, 1575, 1490, 1441, 1353, 1283, 1241, 1123, 1102, 1020, 979, 738; 'H NMR (DMSO-d₆, δ, ppm): 8.23 (d, 1H, J = 7.5 Hz), 7.93 (d, 1H, J = 9.5 Hz), 7.70 (d, 1H, J = 2.5 Hz), 7.57-7.41 (m, 3H), 7.25-7.11 (m, 2H), 4.41 (m, 1H), 4.29 (s, 3H), 4.18 (ABq, 2H), 3.79-3.48 (m, 2H), 3.13 (s, 3H), 2.98-2.80 (m, 1H), 1.90-1.74 (m, 2H), 1.70-1.44 (m, 5H), 1.17 (d, 3H, J = 6.5 Hz); MS (m/e, %): 477 (M⁺, 41), 377 (100); Analysis: for C₂₅H₂₉N₃O₄ + 0.5 H₂O (486.61) calcd.: 69.11% C, 7.45% H, 8.64% N; found: 69.23% C, 7.85% H, 8.80% N.

9-[5-(α-L-Daunosaminloxy)penthox]-5,11-dimethyl-5H-indolo[2,3-b]quinoline (14)

The synthesis was performed using compounds 4 and 10, to give 14 as deep red crystals, yield 784 mg (55%); m.p. 130–132°C.

IR (KBr, cm⁻¹): 3166, 2931, 1635, 1566, 1527, 1494, 1456, 1278, 1205, 1121, 1028, 977, 753; 'H NMR (DMSO-d₆, δ, ppm): 8.32 (d-d, 1H, J = 8 - 1 Hz), 7.95-7.89 (m, 2H), 7.72 (d, 1H, J = 2 Hz), 7.53-7.46 (m, 2H), 7.10 (d-d, 1H, J = 8.5 - 2.5 Hz), 4.71 (d, 1H, J = 2.5 Hz), 4.54-4.30 (m, 1H), 4.24 (s, 3H), 4.07 (ABq, 2H), 3.70 (q, 1H, J = 6.5 Hz), 3.60-3.48 (m, 1H), 3.10 (s, 3H), 2.90 (m, 1H), 1.88-1.70 (m, 2H), 1.63-1.42 (m, 5H), 1.07 (d, 3H, J = 6.5 Hz); MS (m/e, %): 477 (M⁺, 93), 377 (57), 275 (28), 261 (100); Analysis: for C₂₅H₂₉N₃O₄ + H₂O (495.61) calcd.: 67.86% C, 7.52% H, 8.47% N; found: 67.56% C, 7.49% H, 8.13% N.

Antiproliferative assays in vitro

Cell lines: established in vitro, A549 (non-small cell lung cancer), MCF-7 (breast cancer), Hs294T (melanoma) and normal mice fibroblasts (BALB/3T3) were used. Additionally, the cells of the following human cancer lines and their drug-
resistant variants were used for the drug resistance assay: LoVo – colon adenocarcinoma and LoVo/DX – variant resistant to doxorubicin with multidrug cross-resistance. The mechanism of drug resistance depends on the expression of the MDR1 gene product-P-glycoprotein (P-gp), the multidrug resistance-related protein (MRP) and the lung resistance-related protein (LRP).

MES-SA – uterine carcinoma and MES-SA/DX5 – variant resistant to doxorubicin with multidrug cross-resistance. The mechanism of drug resistance depends on the expression of P-gp. HL-60 – promyelocytic leukemia and HL-60/MX2 – variant resistant to mitoxanthrone with multidrug cross-resistance. The mechanism of drug resistance does not depend on the expression of P-gp but seems to be related to the differential activity of topoisomerase II.

All lines were obtained from American Type Culture Collection (Rockville, Maryland, USA) with the exception of HL-60 cell line - from European Type Culture Collection courtesy of Professor Spik and Dr. Mazurier (Laboratory of Biological Chemistry USTL, Lille, France) and LoVo and LoVo/DX courtesy of Prof. E. Borowski (Technical University of Gdańsk, Poland). All cell lines were maintained in the Institute of Immunology and Experimental Therapy, Wrocław, Poland.

A549, MCF-7, LoVo and doxorubicin-resistant subline LoVo/DX, as well as MES-SA and doxorubicin-resistant subline MES-SA/DX5 were cultured in the mixture of RPMI 1640 and Opti-MEM (1 : 1) medium (Gibco, Scotland, UK) supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 5% fetal bovine serum (all from Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The culture of LoVo/DX and MES-SA/DX5 cells was supplemented with 15 nM doxorubicin (from the Institute of Biotechnology and Antibiotics, Warszawa, Poland) and of MCF-7 with 0.01 mg/mL insulin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The human promyelocytic leukemia (HL-60 and resistant to mitoxanthrone subline HL-60/MX2) were cultured in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodium pyruvate, 10% fetal bovine serum. Hs294T and BALB/3T3 in Dulbecco medium supplemented with 2 mM L-glutamine and 1.0 mM sodium pyruvate, 10% fetal bovine serum. The whole culture medium was supplemented with 100 units/mL penicillin, and 100 µg/mL streptomycin (both from Polfa, Tarchomin S.A., Poland). All cell lines were grown at 37°C in the 5% CO2 humidified atmosphere.

Cytotoxicity assay: Test solutions of the tested compounds (1 mg/mL) were prepared by dissolving the substances in 100 µL of the DMSO complemented with 900 µL of the tissue culture medium. Afterwards, the tested compounds were diluted in the culture medium to reach the final concentrations of 100, 10, 1, 0.1, 0.01 and 0.001 µg/mL. Twenty-four hours before the addition of the tested agents, the cells were plated in 96-well plates (Sarstedt, USA) at the density of 10^4 cells per well in 100 µL of the culture medium.

The SRB assay: The details of this technique were described by Skehan et al. (14). The cytotoxicity assay was performed after a 72-h exposure of the cultured cells to varying concentrations (from 0.001

Figure 1. Synthesis of aminosaccharide synthons
to 100 µg/mL) of the tested agents. The cells attached to the plastic were fixed by gently layering cold 50% TCA (trichloroacetic acid, Aldrich-Chemie, Germany) on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed five times with tap water. The background optical density was measured in the wells filled with the culture medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma, Germany) dissolved in 1% acetic acid (POCh, Gliwice, Poland) for 30 min. Unbound dye was removed by rinsing (4×) with 1% acetic acid. The protein-bound dye was extracted with 10 mM non-buffered Tris base (Sigma, Germany) for the determination of optical density (at 540 nm) in a computer-interfaced, 96-well micro titer plate reader Multiskan RC photometer (Labsystems, Helsinki, Finland). Each compound in a given concentration was tested in triplicate in each experiment which was repeated 3-5 times.

The MTT assay: This technique was applied for the cytotoxicity screening against human leukemia.

![Synthesis of 5H-indolo[2,3-b]quinoline O-aminoglycosides](image)

where

\[ \text{All = allyl, Ac = acetyl, pNBz = p-nitrobenzoyl} \]

Figure 2. Synthesis of 5H-indolo[2,3-b]quinoline O-aminoglycosides
cells growing in the suspension culture (15). An assay was performed after a 72-h exposure to varying concentrations (from 0.001 to 100 µg/mL) of the tested agents. For the last 3-4 h of the incubation 20 µL of the MTT solution was added to each well (MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; stock solution: 5 mg/mL, Sigma, Germany). The mitochondria of viable cells reduce the pale yellow MTT to a navy blue formazan: the more viable cells are present in the well, the more MTT will be reduced to formazan. When the incubation time was completed, 80 µL of the lysing mixture was added to each well (lysing mixture: 225 mL dimethylformamide, 67.5 g sodium dodecyl sulfate and 275 mL of distilled water). After 24 h, when formazan crystals had been dissolved, the optical densities of the samples were read on a Multiskan RC photometer at 570 nm wavelength.

Each compound in a given concentration was tested in triplicate in each experiment which was

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Cell line/ IC_{50} ± SD [µg/mL]</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>KB</td>
</tr>
<tr>
<td>11</td>
<td>2.76 ± 0.38</td>
</tr>
<tr>
<td>12</td>
<td>3.06 ± 0.10</td>
</tr>
<tr>
<td>13</td>
<td>1.19 ± 0.55</td>
</tr>
<tr>
<td>14</td>
<td>1.54 ± 0.44</td>
</tr>
<tr>
<td>DIMIQ*</td>
<td>1.14 ± 0.61</td>
</tr>
</tbody>
</table>

IC_{50} – compound concentration leading to 50% inhibition of cell proliferation; *DIMIQ – referential compound 5,11-dimethyl-indolo[2,3-b]quinoline.
repeated 3-5 times. The results of the cytotoxic activity in vitro were expressed as IC50, the concentration of a compound (in µM) that inhibits the proliferation rate of the tumor cells by 50% as compared to the control untreated cells.

Using the obtained IC50 values, resistance indexes (RI) were calculated by dividing the IC50 values of the compounds tested against the cells of the drug resistant cell subline by respective values obtained against the cells of the drug sensitive cell line. According to Harker et al. (16), three categories of the cells could be distinguished: (a) the cells are drug-sensitive - if the ratio approaches 0-2; (b) the cells are moderately drug-resistant - if the ratio ranges from 2 to 10; (c) the cells are markedly drug-resistant - if the ratio is higher than 10.

RESULTS AND DISCUSSION

Synthesis

Aminosaccharide synths 9 and 10 were prepared as shown in Figure 1. Starting compounds 5 and 7 were synthesized as described in the literature (12). Allyloxycarbonylamine substituents at 4-positions in 5 and 7 were firstly transformed into 4-trifluoroacetamide ones by consecutive reactions with tetrakis(triphenylphosphine)palladium and the formic acid in THF and then with trifluoroacetic acid in the presence of triethylamine to give intermediate compounds 6 and 8. By reacting 6 and 8 with the corresponding bromoalkohols in the presence of triphenylphosphine hydrobromide in dichloromethane, final O-bromoalkyl aminoglycosides 9 and 10 were obtained.

The 5H-indolo[2,3-b]quinolinols 3 and 4 were manufactured starting from 2- or 9- methoxy derivatives of 6H-indolo[2,3-b]quinoline 1 or 2, synthesized according to the literature procedures (13). Compounds 1 or 2 were quaternized with methyl sulfate, then transformed into corresponding 2- or 9-hydroxy derivatives in the reaction with HBr in the acetic acid to afford 3 or 4 isolated after basification with the ammonia solution. All compounds tested were synthesized in the reactions outlined in Figure 2. The condensation of 5H-indolo[2,3-b]quinolinols 3 and 4 with O-bromoalkyl aminoglycosides 9 and 10 in the presence of sodium hydride in the DMF, followed by the de-protection of the obtained intermediates by stirring with potassium carbonate in the chloroform-methanol mixture afforded final products 11–14. These compounds were isolated by extraction, purified by column chromatography and used for bio-tests.

The obtained series of 5H-indolo[2,3-b]quinoline O-aminoglycosides 11–14 provided good materi-
al to verify in a biological assay our assumptions concerning the influence of such factors on the activity, as the kind and the position of the aminoglycosyl substituent at the indoloquinoline core (C-2, C-9) and the distance between the aminoglycosyl substituent and the indoloquinoline chromophore (linkers of two different lengths). However, the most important task was to examine the influence of changing the indoloquinoline chromophore from a 6H- isomer (12) to a 5H- one. The structures of indoloquinoline O-aminoglycosides tested are given in Table 1.

**Biological assays**

**Anti-proliferative assay in vitro**

First, we have performed a preliminary screening of the obtained 5H-indolo[2,3-b]quinoline O-aminoglycosides and O-aminoglycosides on the KB-cells to eliminate the derivatives showing low cytotoxic properties. All compounds 11-14 appeared to be active (with IC₅₀ lower than 4 µg/mL against KB cells), so then anti-proliferative studies were carried out for those four compounds against lung adenocarcinoma A549, breast cancer MCF-7, melanoma Hs294T human cell lines and mice fibroblasts BALB/3T3 (Table 2).

The cytotoxicity of all compounds was expressed as IC₅₀ values which were in the range 0.28–3.06 µg/mL. The cytotoxicity of the tested derivatives was comparable to the referential DIMIQ (IC₅₀ between 0.38-2.29 µg/mL). However, the tested compounds were more potent than their analogues containing a 6H-isomeric indoloquinoline chromophore (IC₅₀ between 2.3-3.4 µg/mL) (12). All tested compounds 11-14 expressed the cytotoxicity comparable to normal (BALB/3T3 mice fibroblast) and cancer cells.

**Ability to overcome the barrier of multidrug resistance**

Two compounds, namely 12 and 14, were examined for their ability to overcome the barrier of multidrug resistance (MDR). In our research we used three various human cancer cell lines and their drug-resistant sublines: human colon cancer (LoVo) and doxorubicin-resistant LoVo/DX (P-gp-dependent, MRP-, LRP-dependent multidrug resistance), uterine sarcoma (MES-SA) and MES-SA/DX5 (P-gp-dependent resistance to doxorubicin), human promyelocytic leukemia cell line (HL-60) and HL-60/MX2 (P-gp-independent and topoisomerase II-dependent resistance). The results are summarized in Table 3.

The resistance indexes (RI) were calculated by dividing the IC₅₀ values of the compounds tested against the cells of the drug resistant cell sub-lines by the respective values obtained against the cells of the drug sensitive cell lines. As we have found out, both compounds can overcome only the barrier of P-gp-independent and topoisomerase II-dependent resistance (HL-60/MX2) (RI < 2). However, human cancer cells lines P-gp-dependent, MRP-, LRP-dependent multidrug resistant (LoVo/DX) and P-gp-dependent resistant to doxorubicin (MES-SA/DX5) were also resistant to compounds 12 and 14 (RI > 10).

**CONCLUSION**

The aim of this study was the synthesis of novel 5H-indolo[2,3-b]quinoline O-aminoglycosides in order to check our hypothesis that the construction of hybrids composed of the active 5H-indolo[2,3-b]quinoline chromophore and daunosaminyl or acosaminyl moiety, attached to the alkyl or alkoxy chain, may result in the cytotoxic activity of the obtained derivatives that is much higher than in the case of the parent DIMIQ (5,11-dimethyl-5H-indolo[2,3-b]quinoline) and 6H-indoloquinoline O-aminoglycosides (12). Moreover, we wanted to obtain material which could be used to verify, in a biological assay, our assumptions concerning the relationship between the activity and the structure of the obtained compounds.

In the preliminary screening on the KB-cells, all compounds (11-14) were selected for further examination. Actually, novel 5H-indolo[2,3-b]indoloquinoline O-aminoglycosides 11, 12, 13 and 14 showed the anti-proliferative activity in vitro against human lung adenocarcinoma A549, breast cancer MCF-7, melanoma Hs294T (Table 2) as well as against promyelocytic leukemia HL-60, uterine sarcoma MES-SA and colon cancer LoVo cell lines (Table 3). As we expected, their activity was, in general, 10 times higher than that of the 6H-analogs (12) and comparable to the one of the referential DIMIQ. Unexpectedly it appeared that except for HL-60/MX2 (P-gp-independent and topoisomerase II-dependent resistance), other MDR tumor cell lines (LoVo/DX, P-gp-dependent, MRP-, LRP-dependent multidrug resistance) and MES-SA/DX5 (P-gp-dependent resistance to doxorubicin) are also resistant to the 5H-indolo[2,3-b]indoloquinoline O-aminoglycosides (12 and 14) tested. This is surprising because 6H-analogs, in general 10 times less active against non-MDR tumor cell lines, as well as the DIMIQ itself, are able to overcome drug resistance in all examined MDR cell lines.

The cytotoxicity of the tested compounds against tumor cell lines and against normal cells (mice fibroblasts BALB/3T3) was comparable.
REFERENCES


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The introduction of a halogen group in a strategic position of a molecule appeared to be a very powerful and versatile tool for the development of compounds with biological activities. The insertion of electron withdrawing fluorine and chlorine atoms into a drug modulates the steric and electronic parameters that leads to increased lipophilicity, thus enhances rates of absorption and transport of drugs in vivo (1). The presence of a halogen has an influence on the properties of functional groups nearby, exerting the dipole moment of the molecule, changing its acidity or basicity, as well as reactivity (2).

Literature survey reveals that molecules composed of thiourea system, substituted with fluoro- or chlorophenyl terminal fragment, show a broad spectrum of antimicrobial activities. Para-substituted halogeno derivatives of that group display significant inhibitory activity against Gram-positive cocci and Gram-negative bacteria (3-5). They also have become a subject of interest due to their antifungal (1, 3, 4) and antimycobacterial (6) activities. Some heterocyclic thiourea derivatives carrying a halogen substituent at ortho or meta position of a phenyl ring have also attracted attention as potential antimicrobial agents (3, 7-10). In addition, thiourea derivatives with halogen groups have been reported as potent anti-inflammatory agents, acting as inducible nitric oxide synthase (iNOS) inhibitors (9) or against the pro-inflammatory cytokines (3, 10). On the other hand, antiviral activity of a compound can be attributed to the presence of thiourea core bearing electron-withdrawing functionalities like F, Cl, CF₃ at para position of a benzene ring (11, 12). This group of derivatives was also found to be cytotoxic and express antiproliferative activity (11, 13). It was established that the class of cyclic thiourea derivatives containing halogen groups share antimicrobial activity of their linear analogs (13-15). What is more, antidiabetic properties of para-chloro substituted derivatives were reported (4).

Thiourea derivatives of 4-azatricyclo[5.2.2.0²,6]undec-8-ene-3,5-dione – synthesis and biological activity

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Abstract: A series of halogen derivatives of thiourea bearing a polycyclic imide core has been efficiently synthesized and evaluated for antimicrobial activity. The structures of the compounds were established by ¹H and ¹³C NMR and MS methods. The molecular structure of 4Clc was determined by an X-ray crystallography. Compounds containing 3-chloro-4-fluorophenyl substituent (3Cl4Fb, 3Cl4Fd) were found to be the most promising against Gram-positive bacteria (MIC values ranged from 8 to 32 µg/mL for standard and 32-64 µg/mL for hospital strains). The in vitro cytotoxicity against MT-4 cells of all compounds was evaluated.

Keywords: synthesis, thiourea derivatives, antimicrobial activity, cytotoxicity
ranging pharmacological activities, such as antibacterial (7, 16-18), antifungal (7, 16, 17) and antiviral (19, 20). Derivatives of 4-azatricyclo[5.2.2.02,6]undec-8-ene-3,5-dione are also known as the 5-HT\textsubscript{2} receptor ligands (19, 21).

Motivated by the afore-mentioned literature and continuing our earlier work on different polycyclic imide derivatives bearing halogen-containing thiourea moieties (7), we envision our approach towards the design and synthesis of derivatives incorporating these nuclei for their antimicrobial and cytotoxic activity.

EXPERIMENTAL

Chemistry

All reagents, solvents, and starting materials were obtained from commercial suppliers and used without further purification. Melting points were determined in a Kofler's apparatus and are uncorrected. The NMR spectra were recorded on Varian VNMR 300 Oxford NMR spectrometer, operating at 300 MHz (\textit{H} NMR, relax. delay 1.000 s, pulse 30.0 degrees) and 75.4 MHz (\textit{C} NMR, relax. delay 3.700 s, pulse 45.0 degrees, Waltz-16 modulated). Chemical shifts (\textit{\Delta}) were expressed in parts per million relative to tetramethylsilane used as the internal reference. Mass spectral ESI measurements were carried out on Waters ZQ Micro-mass instrument with quadruple mass analyzer. The spectra were performed in the negative ion mode at a declustering potential of 40-60 V. The sample was previously separated on a UPLC column (C18) using UPLC ACQUITY system by Waters connected with DPA detector. Flash chromatography was performed on Merck silica gel 60 (200-400 mesh) using chloroform eluent. Analytical TLC was carried out on silica gel F\textsubscript{254} (Merck) plates (0.25 mm thickness).

The intensities of diffraction reflections for 4Clc were measured at 296 K with a KM4 diffractometer, using graphite monochromated Cu K\textalpha\ radiation (\lambda = 1.54178 Å) and \alpha/2\theta scan mode. Crystal structure was solved by the SHELXS-97 program and refined by full-matrix least squares on \textit{F}\textsuperscript{2} using the SHELXL-97 program (22). All non-hydrogen atoms were refined with anisotropic displacement parameters. Hydrogen atoms were positioned geometrically and allowed to ride on their parent atoms, with \textit{U}_{\text{iso}}(\textit{H}) = 1.2 \textit{U}_{\text{eq}}(\textit{C, N}). The experimental details and final atomic parameters have been deposited with the Cambridge Crystallographic Data Centre as supplementary material (CCDC No. 1028170). Copies of the data can be obtained free of charge on request via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

General procedure for the synthesis of thiourea derivatives of 4-azatricyclo[5.2.2.02,6]undec-8-ene-3,5-dione

A solution of 4-amino-tricyclic imide (0.0025 mol) in acetonitrile (10 mL) was treated with appropriate isothiocyanate (0.003 mol) and the mixture was refluxed for 18 h. Then, the solvent was removed on rotary evaporator. The residue was purified by column chromatography (chloroform : methanol; 9:5:0.5 v/v). The compound was crystalized from ethanol.

1-(2-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl)thiourea (2Cla)

Yield 82\%, m.p. 138°C. H NMR (CDCl\textsubscript{3}, \textit{\Delta}, ppm): 1.47 (s, 6H, CH\textsubscript{3}), 1.54 (s, 6H, CH\textsubscript{3}), 1.42-1.47 (m, 2H, CH\textsubscript{2}), 3.13 (s, 2H, CH), 7.14-7.28 (m, 2H, CH\textsubscript{arom.}), 7.39-7.43 (m, 2H, CH\textsubscript{arom.}), 7.95 (br. s, 1H, NH), 8.24 (br. s, 1H, NH). \textit{\textit{\textsuperscript{13}C} NMR (DMSO-d\textsubscript{6}, \textit{\Delta}, ppm): 10.8, 16.5, 50.8, 54.0, 62.5, 79.1, 119.8, 122.5, 128.4, 128.5, 136.6, 138.1, 174.5, 181.5. HRMS (ESI): calc. for C\textsubscript{20}H\textsubscript{22}ClN\textsubscript{3}S \textit{[M - H]}\textsuperscript{-}: 402.5893, found: 402.5885.

1-(3-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl)thiourea (3Cla)

Yield 72\%, m.p. 167°C. H NMR (CDCl\textsubscript{3}, \textit{\Delta}, ppm): 1.4-1.49 (m, 2H, CH\textsubscript{2}), 1.47 (s, 6H, CH\textsubscript{3}), 1.54 (s, 6H, CH\textsubscript{3}), 1.4-1.51 (m, 2H, CH\textsubscript{2}), 3.13 (s, 2H, CH), 7.15-7.31 (m, 3H, CH\textsubscript{arom.}), 7.45 (s, 1H, CH\textsubscript{arom.}), 8.36 (br. s, 1H, NH), 8.54 (br. s, 1H, NH). \textit{\textit{\textsuperscript{13}C} NMR (DMSO-d\textsubscript{6}, \textit{\Delta}, ppm): 10.8, 16.5, 50.8, 54.0, 62.4, 124.1, 124.1, 126.3, 126.4, 128.6, 130.6, 136.7, 173.8, 181.5. HRMS (ESI): calc. for C\textsubscript{20}H\textsubscript{22}ClN\textsubscript{3}O\textsubscript{2}S \textit{[M - H]}\textsuperscript{-}: 402.5893, found: 402.5885.

1-(4-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl)thiourea (4Cla) has been synthesized as described previously (23).

1-(2-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl)thiourea (2Clb)

Yield 78\%, m.p. 134°C. H NMR (CDCl\textsubscript{3}, \textit{\Delta}, ppm): 0.62 (d, 3H, CH\textsubscript{3}, \textit{J} = 6.3 Hz), 1.36 (s, 6H,
CH\textsubscript{3}), 1.49 (s, 6H, CH\textsubscript{3}), 1.46–1.62 (m, 1H, CH), 3.05 (s, 2H, CH), 7.19–7.33 (m, 2H, CH\textsubscript{arom}), 7.43–7.46 (m, 1H, CH\textsubscript{arom}), 7.52 (br. s, 1H, NH), 7.76 (br. s, 1H, NH) 7.82–7.85 (m, 1H, CH\textsubscript{arom}). ¹³C NMR (DMSO-d\textsubscript{6}, δ, ppm): 7.1, 11.1, 14., 51.1, 56.6, 63.5, 115.9, 124.1, 126.5, 128.6, 130.6, 132.9, 137.8, 181.4. HRMS (ESI): calc. for C\textsubscript{21}H\textsubscript{24}ClN\textsubscript{3}O\textsubscript{2}S [M - H]: 416.1189, found: 416.1200.

1-(3-Chlorophenyl)-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.2.0\textsuperscript{2,6}\]undec-8-en-4-yl) thiourea (3Clb)

Yield 82%, m.p. 154°C. ¹H NMR (CDCl\textsubscript{3}, δ, ppm): 0.62 (d, 3H, CH\textsubscript{3}, J = 6.3 Hz), 1.36 (s, 6H, CH\textsubscript{3}); 1.49 (s, 6H, CH\textsubscript{3}); 1.52–1.62 (m, 1H, CH); 3.05 (s, 2H, CH), 7.21–7.3 (m, 4H, CH\textsubscript{arom}); 7.42 (br. s, 1H, NH); 7.97 (br. s, 1H, NH). ¹³C NMR (DMSO-d\textsubscript{6}, δ, ppm): 7.1, 11.2, 14.4, 51.1, 56.6, 63.6, 115.9, 124.8, 126.5, 128.6, 130.6, 132.9, 137.8, 174.4, 181.9. HRMS (ESI): calc. for C\textsubscript{21}H\textsubscript{24}ClN\textsubscript{3}O\textsubscript{2}S [M - H]: 416.1189, found: 416.1200.

1-(4-Chlorophenyl)-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-aza-tricyclo[5.2.2.0\textsuperscript{2,6}\]undec-8-en-4-yl) thiourea (4Clb)

Yield 85%, m.p. 159°C. ¹H NMR (CDCl\textsubscript{3}, δ, ppm): 0.62 (d, 3H, CH\textsubscript{3}, J = 6.3 Hz), 1.35 (s, 6H, CH\textsubscript{3}), 1.48 (s, 6H, CH\textsubscript{3}), 1.53–1.62 (m, 1H, CH), 3.05 (s, 2H, CH), 7.25 (s, 2H, CH\textsubscript{arom}), 7.32 (s, 2H, CH\textsubscript{arom}), 7.72 (br. s, 1H, NH), 7.98 (br. s, 1H, NH); ¹³C NMR (DMSO-d\textsubscript{6}, δ, ppm): 7.0, 11.1, 14.3, 51.1, 56.8, 63.9, 115.9, 124.1, 126.3, 128.6, 130.6, 132.9, 137.9, 174.3, 181.8. HRMS (ESI): calc. for C\textsubscript{21}H\textsubscript{24}ClN\textsubscript{3}O\textsubscript{2}S [M - H]: 416.1189, found: 416.1200.

1-(2-Chlorophenyl)-3-(1-30-isopropyl-8-methyl-3,5-dioxo-4-aza-tricyclo[5.2.2.0\textsuperscript{2,6}\]undec-8-en-4-yl) thiourea (2Clc)

Yield 87%, m.p. 155°C. ¹H NMR (CDCl\textsubscript{3}, δ, ppm): 0.98 (d, 3H, CH\textsubscript{3}, J = 6.9 Hz), 1.10 (d, 3H, CH\textsubscript{3}, J = 6.6 Hz), 1.25–1.35 (m, 2H, CH\textsubscript{3}), 1.42–1.54 (m, 2H, CH\textsubscript{3}), 1.49 (s, 3H, CH\textsubscript{3}), 2.51–2.60 (m, 1H, CH), 2.75 (d, 1H, CH, J = 8.4 Hz), 3.12 (d, 1H, CH, J = 8.1 Hz), 5.98 (dd, 2H, CH\textsubscript{arom}), 7.43–7.46 (m, 1H, CH\textsubscript{arom}), 7.89 (d, 1H, CH\textsubscript{arom}, J = 8.1 Hz), 7.6 (br. s, 1H, NH), 7.04 (br. s, 1H, NH). ¹³C NMR (DMSO-d\textsubscript{6}, ppm): 16.8, 18.2, 22.3, 29.1, 33.5, 36.2, 42.9, 44.2, 47.8, 127.3, 128.6, 129.5, 135.1, 135.3, 136.1, 136.4, 173.8, 181.9. HRMS (ESI): calc. for C\textsubscript{21}H\textsubscript{24}ClN\textsubscript{3}O\textsubscript{2}S [M - H]: 416.1189, found: 416.1200.
1-(5-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.0^2,6]dec-8-en-4-yl)-thiourea (4Cl)

Yield 82%, m.p. 169°C. 'H NMR (CDCl_3, δ, ppm): 1.47 (s, 6H, CH_3), 1.54 (s, 6H, CH_3), 1.38–1.58 (m, 2H, CH_3), 1.34 (dd, 2H, CH, J = 5.8 Hz), 1.70–1.75 (m, 2H, CH_2), 0.86 (br. s, 1H, NH). 13C NMR (CDCl_3, δ, ppm): 7.0, 11.1, 14.3, 51.2, 54.6, 63.5, 72.1, 115.7, 115.9, 124.1, 126.49, 128.6, 130.6, 136.7, 173.9, 181.4. HRMS (ESI): calc. for C_{13}H_{19}FNO_2S [M - H]: 386.1347, found: 386.1339.

1-(3-Chloro-4-fluorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.0^2,6]dec-8-en-4-yl)-thiourea (3Cl)

Yield 89%, m.p. 167°C. 'H NMR (CDCl_3, δ, ppm): 0.62 (d, 3H, CH_3, J = 6.3 Hz), 1.32 (s, 6H, CH_3), 1.51 (s, 6H, CH_3), 1.51–1.65 (m, 1H, CH), 8.22 (br. s, 1H, NH). 13C NMR (CDCl_3, δ, ppm): 7.0, 11.1, 14.3, 51.1, 56.7, 63.5, 115.7, 124.0, 126.4, 128.6, 130.6, 132.9, 137.8, 174.1, 181.4. HRMS (ESI): calc. for C_{14}H_{21}FNNO_2S [M - H]: 400.1508, found: 400.1495.

1-(5-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.0^2,6]dec-8-en-4-yl)-thiourea (4Cl)

Yield 80%, m.p. 153°C. 'H NMR (CDCl_3, δ, ppm): 0.62 (d, 3H, CH_3, J = 6.3 Hz), 1.35 (s, 6H, CH_3), 1.51 (s, 6H, CH_3), 1.51–1.65 (m, 1H, CH), 8.22 (br. s, 1H, NH). 13C NMR (CDCl_3, δ, ppm): 7.0, 11.1, 14.3, 51.1, 56.7, 63.5, 115.7, 124.0, 126.4, 128.6, 130.6, 132.9, 137.8, 174.1, 181.4. HRMS (ESI): calc. for C_{13}H_{17}FNOO_2S [M - H]: 386.1347, found: 386.1339.

1-(6-Chlorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.0^2,6]dec-8-en-4-yl)-thiourea (4Cl)

Yield 91%, m.p. 139°C. 'H NMR (CDCl_3, δ, ppm): 1.48 (s, 6H, CH_3), 1.52 (s, 6H, CH_3), 1.42–1.57 (m, 2H, CH_3), 3.013 (s, 2H, CH), 7.05 (t, 2H, CH, J = 6.3 Hz), 7.32–7.35 (m, 2H, CH), 7.68 (br. s, 1H, NH), 7.92 (br. s, 1H, NH). 13C NMR (CDCl_3, δ, ppm): 10.8, 16.5, 51.4, 53.9, 62.8, 115.9, 126.4, 128.6, 130.7, 136.7, 172.9, 180.9. HRMS (ESI): calc. for C_{15}H_{17}FNOO_2S [M - H]: 400.1508, found: 400.1495.
Thiourea derivatives of...

1-(3-Fluorophenyl)-3-(10-isopropyl-8-methyl-3,5-dioxo-4-aza-tricyclo[5.2.2.0\(^2,6\)]undec-8-en-4-yl)thiourea (3Fc)

Yield 78%, m.p. 162°C. \( \delta \) NMR (CDCl\(_3\), ppm): 0.81 (d, 3H, CH\(_3\), J = 6.3 Hz), 0.91 (d, 3H, CH\(_3\), J = 6.6 Hz), 1.07–1.14 (m, 2H, CH\(_2\)), 1.34–1.4 (m, 1H, CH), 1.70 (s, 3H, CH\(_3\)), 1.81–1.87 (m, 1H, CH\(_2\)), 2.93–3.02 (m, 3H, CH), 3.23 (d, 1H, CH), 5.72 (d, 1H, CH\(_=\), J = 5.1 Hz), 6.97 (t, 1H, CH\(_{arom.}\), J = 6.3 Hz), 7.18 (d, 1H, CH\(_{arom.}\), J = 8.1 Hz), 7.23 (d, 1H, CH\(_{arom.}\), J = 8.1 Hz), 7.35 (q, 1H, CH\(_{arom.}\), J = 5.7 Hz), 7.72 (br. s, 1H, NH), 8.09 (br. s, 1H, NH). \( ^{13} \)C NMR (DMSO-d\(_6\), ppm): 20.3, 20.6, 20.8, 30.1, 32.6, 34.7, 37.4, 41.7, 43.9, 116.1, 121.6, 124.2, 126.2, 128.2, 130.7, 131.0, 175.3, 181.7. HRMS (ESI): calc. for C\(_{21}\)H\(_{24}\)FN\(_3\)O\(_2\)S [M - H] -: 400.1508, found: 400.1495.

1-(4-Fluorophenyl)-3-(1-isopropyl-7-methyl-3,5-dioxo-4-aza-tricyclo[5.2.2.0\(^2,6\)]dec-8-en-4-yl)thiourea (4Fd) has been synthesized as described previously (19).

1-(3-Chloro-4-fluorophenyl)-3-(1,7,8,9-tetramethyl-3,5-dioxo-4-azatricyclo[5.2.1.0\(^2,6\)]dec-8-en-4-yl)thiourea (3Cl4Fa)

Yield 85%, m.p. 123°C. \( \delta \) NMR (CDCl\(_3\), ppm): 1.4 (s, 6H, CH\(_3\)), 1.52 (s, 6H, CH\(_3\)), 1.25–1.59 (m, 2H, CH\(_2\)), 3.12 (s, 2H, CH), 7.08–7.28 (m, 1H, CH\(_{arom.}\)), 7.28 (d, 1H, CH\(_{arom.}\), J = 4.5 Hz), 7.57 (d, 1H, CH\(_{arom.}\), J = 4.5 Hz), 7.95 (br. s, 1H, NH), 8.24 (br. s, 1H, NH). \( ^{13} \)C NMR (DMSO-d\(_6\), ppm): 10.8, 16.5, 50.8, 54.0, 62.4, 119.8, 123.9, 128.1, 128.3, 129.1, 136.6, 138.1, 174.5, 181.5. HRMS (ESI): calc. for C\(_{23}\)H\(_{24}\)ClFN\(_3\)O\(_2\)S [M - H] -: 420.0958, found: 420.0949.

1-(3-Chloro-4-fluorophenyl)-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-azatricyclo[5.2.2.0\(^2,6\)]dec-8-en-4-yl)thiourea (3Cl4Fb)

Yield 82%, m.p. 161°C. \( \delta \) NMR (CDCl\(_3\), ppm): 0.59 (d, 3H, CH\(_3\), J = 6.9 Hz), 1.3 (s, 6H, CH\(_3\)), 1.49 (s, 6H, CH\(_3\)), 1.42–1.62 (m, 1H, CH), 3.04 (s, 2H, CH), 7.17 (t, 1H, CH\(_{arom.}\), J = 8.7 Hz), 7.28–7.3 (m, 1H, CH\(_{arom.}\)), 7.57 (d, 1H, CH\(_{arom.}\), J = 4.5 Hz), 7.85 (br. s, 1H, NH), 7.94 (br. s, 1H, NH). \( ^{13} \)C NMR (DMSO-d\(_6\), ppm): 16.8, 18.2, 22.4, 29.2, 33.5, 36.2, 42.9, 44.2, 47.8, 127.3, 128.6, 129.5, 135.3, 136.4, 174.1, 181.8. HRMS (ESI): calc. for C\(_{23}\)H\(_{24}\)ClFN\(_3\)O\(_2\)S [M - H] -: 434.1095, found: 434.1105.

Yield 78%, m.p. 136°C. \( \delta \) NMR (CDCl\(_3\), ppm): 0.94 (d, 3H, CH\(_3\), J = 6.9 Hz), 1.06 (d, 3H, CH\(_3\), J = 6.9 Hz), 1.1–1.25 (m, 2H, CH\(_3\)), 1.39–1.61 (m, 2H, CH\(_3\)), 1.4 (s, 3H, CH\(_3\)), 2.8 (d, 1H, CH\(_=\), J = 7.8 Hz), 3.1 (d, 1H, CH\(_=\), J = 7.8 Hz), 3.41–3.48 (m, 1H, CH), 5.95 (dd, 2H, CH\(_=\), J = 10.8 Hz), 7.01 (t, 1H, CH\(_{arom.}\), J = 7.6 Hz), 7.28 (d, 1H, CH\(_{arom.}\), J = 8.1 Hz), 7.37 (dd, 2H, CH\(_{arom.}\), J = 7.7 Hz), 8.30 (br. s, 1H, NH), 10.2 (br. s, 1H, NH). \( ^{13} \)C NMR (DMSO-d\(_6\), ppm): 16.8, 18.2, 22.4, 29.1, 33.4, 36.2, 42.7, 44.3, 47.7, 127.3, 128.8, 128.9, 135.3, 136.4, 174.1, 181.8. HRMS (ESI) calc. for C\(_{21}\)H\(_{24}\)FN\(_3\)O\(_2\)S [M - H] -: 400.1508, found: 400.1495.
CH, J = 6 Hz), 5.66 (d, 1H, CH=, J = 6 Hz), 7.20–7.33 (m, 2H, CH
 arom.), 7.61–7.64 (m, 1H, CH arom.), 7.86 (br. s, 1H, NH), 7.98 (br. s, 1H, NH).

13C NMR (DMSO-d6, δ, ppm): 20.3, 20.6, 20.8, 30.1, 32.7, 34.6, 37.3, 41.2, 43.9, 116.3, 118.6, 121.2, 135.8, 141.0, 175.8, 191.9. HRMS (ESI): calc. for C21H23ClFN3O2S [M - H]−: 434.1095, found: 434.1105.

1-(3-Chloro-4-fluorophenyl)-3-(1-isopropyl-7-methyl-3,5-dioxo-4-aza-tricyclo[5.2.2.02,6]-undec-8-en-4-yl)-thiourea (3C4Fd)

Yield 76%, m.p. 105°C. 1H NMR (CDCl3, δ, ppm): 0.95 (d, 3H, CH3, J = 6.9 Hz), 1.05 (d, 3H, CH3, J = 6.6 Hz), 1.18–1.3 (m, 2H, CH2), 1.39–1.42 (m, 2H, CH2), 1.42 (s, 3H, CH3), 2.48–2.61 (m, 1H, CH), 2.76 (d, 1H, CH, J = 8.4 Hz), 3.15 (d, 1H, CH, J = 8.1 Hz), 5.98 (d, 2H, CH=, J = 8.1 Hz), 7.17 (t, 1H, CH arom., J = 9 Hz), 7.27–7.32 (m, 1H, CH arom.), 7.58–7.59 (m, 1H, CH arom.), 7.87 (br. s, 1H, NH), 7.98 (br. s, 1H, NH). 13C NMR (DMSO-d6, δ, ppm): 16.7, 18.1, 22.3, 22.4, 29.1, 33.4, 36.1, 42.8, 44.7, 48.2, 116.7, 118.9, 124.9, 126.1, 135.1, 136.4, 153.1, 174.4, 181.2. HRMS (ESI): calc. for C21H23ClFN3O2S [M - H]−: 434.1095, found: 434.1105.

Biology
Antimicrobial studies

All types of microorganisms used in experiments were described previously (19). Antibacterial activity was performed by the disc-diffusion method under standard conditions using Mueller-Hinton II agar medium (Becton Dickinson) according to CLSI (previously NCCLS) guidelines (24). Antifungal activities were examined using Mueller-Hinton agar + 2% glucose and 0.5 µg/mL Methylene Blue Dye Medium (25). Minimal inhibitory concentration (MIC) was tested by the twofold serial microdilution method (in 96-well microtiter plates) using Mueller-Hinton broth medium (Beckton Dickinson) for bacteria or RPMI-1640 medium for Candida species consistent with CLSI guidelines (26, 27).

Cytotoxicity and antiviral assays

CD4+ human T-cells containing an integrated HTLV-1 genome (MT-4) were purchased from American Type Culture Collection (ATCC).

\[
\begin{align*}
\text{R} & = \text{H (compounds a)}, \text{CH}_3 (\text{compounds b}) \\
\text{R} & = \text{2-chlorophenyl (2Cl}_a, \text{2Cl}_b, \text{2Cl}_c, \text{2Cl}_d), \text{3-chlorophenyl (3Cl}_a, \text{3Cl}_b, \text{3Cl}_c, \text{3Cl}_d), \text{4-chlorophenyl (4Cl}_a, \text{4Cl}_b, \text{4Cl}_c, \text{4Cl}_d), \text{2-fluorophenyl (2F}_a, \text{2F}_b, \text{2F}_c, \text{2F}_d), \text{3-fluorophenyl (3F}_a, \text{3F}_b, \text{3F}_c, \text{3F}_d), \text{4-fluorophenyl (4F}_a, \text{4F}_b, \text{4F}_c, \text{4F}_d), \text{3-chloro-4-fluorophenyl (3Cl4F}_a, \text{3Cl4F}_b, \text{3Cl4F}_c, \text{3Cl4F}_d) \\
\end{align*}
\]

Scheme 1. Synthetic procedure for compounds a, b, c and d. (i) furan, benzene, reflux; (ii) hydrazine hydrate 80%, reflux; (iii) substituted phenyl isothiocyanate, acetonitrile, reflux.
Laboratory strain IIIB of human immunodeficiency virus type-1 (HIV-1) was obtained from the supernatant of the persistently infected H9/IIIB cells (NIH 1983).

Compound activity against HIV-1 was based on inhibition of virus-induced cytopathogenicity in MT-4 cell acutely infected with a multiplicity of infection (m.o.i.) of 0.01, as described in Drzewiecka et al. (28). After a 4-day incubation at 37°C, cell viability was determined by the MTT method (29). Efavirenz was used as reference inhibitor.

Cell lines derived from human hematological tumors [CD4+ human acute T-lymphoblastic leukemia (CCRF-CEM), human splenic B-lymphoblastoid cells (WIL-2NS), human acute B-lymphoblastic leukemia (CCRF-SB)] were seeded at an initial density of $1 \times 10^5$ cells/mL in 96 well plates in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 units/mL penicillin G and 100 µg/mL streptomycin.

Cell lines derived from human solid tumors [skin melanoma (SK MEL28), prostate carcinoma...]

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Table 1. Activity of obtained compounds against Gram-positive bacteria and fungi – diameter of growth inhibitory zone (GIZ, mm, applied 400 µg per disc) and minimal inhibitory concentrations (MIC, µg/mL).

<table>
<thead>
<tr>
<th></th>
<th>2Cla</th>
<th>3Clc</th>
<th>3Cl4Fb</th>
<th>3ClFd</th>
<th>Ref.</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em> NCTC 4163</td>
<td>-</td>
<td>-</td>
<td>13 (16)</td>
<td>13 (8)</td>
<td>26 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>-</td>
<td>-</td>
<td>14 (16)</td>
<td>13 (8)</td>
<td>26 (0.5)</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 6538</td>
<td>-</td>
<td>-</td>
<td>13 (16)</td>
<td>16 (8)</td>
<td>28 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 29213</td>
<td>-</td>
<td>-</td>
<td>13 (16)</td>
<td>14 (8)</td>
<td>22 (0.5)</td>
<td>-</td>
</tr>
<tr>
<td><em>S. epidermidis</em> ATCC 12228</td>
<td>-</td>
<td>-</td>
<td>16 (16)</td>
<td>14 (16)</td>
<td>30 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>S. epidermidis</em> ATCC 35984</td>
<td>-</td>
<td>-</td>
<td>13 (32)</td>
<td>12 (16)</td>
<td>32 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>B. subtilis</em> ATCC 6633</td>
<td>-</td>
<td>-</td>
<td>14 (16)</td>
<td>14 (8)</td>
<td>38 (&lt;0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>B. cereus</em> ATCC 11778</td>
<td>-</td>
<td>-</td>
<td>11 (32)</td>
<td>-</td>
<td>26 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>M. luteus</em> ATCC 10240</td>
<td>-</td>
<td>-</td>
<td>18 (32)</td>
<td>19 (8)</td>
<td>24 (1)</td>
<td>-</td>
</tr>
<tr>
<td><em>M. luteus</em> ATCC 9341</td>
<td>-</td>
<td>-</td>
<td>21 (32)</td>
<td>17 (8)</td>
<td>26 (0.25)</td>
<td>-</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>12 (128)</td>
<td>20 (128)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20 (0.5)</td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 90028</td>
<td>-</td>
<td>19 (128)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>29 (0.5)</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> ATCC 22019</td>
<td>12 (128)</td>
<td>19 (128)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25 (0.5)</td>
</tr>
</tbody>
</table>

- Lack of the growth inhibition area. ’Ref. – ciprofloxacin (GIZ – 5 µg/ 9 mm disc), ’Ref. – fluconazole (GIZ – 25 µg/ 9 mm disc).
(DU145)] were also seeded at $1 \times 10^5$ cells/mL in 96 well plates in specific media supplemented with 10% FCS and antibiotics, as above. Cell cultures were then incubated at 37°C in a humidified, 5% CO₂ atmosphere in the absence or presence of serial dilutions of test compounds. Cell viability was determined after 96 h at 37°C by the MTT method.

RESULTS AND DISCUSSION

Chemistry

The preparation and structure of new twenty eight halogeno derivatives of thiourea of 1,7,8,9-tetramethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione, 1,7,8,9,10-pentamethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione, 10-isopropyl-8-methyl-4-azatricyclo[5.2.2.0²,6]undec-8-ene-3,5-dione and 1-isopropyl-7-methyl-4-azatricyclo[5.2.2.0²,6]undec-8-ene-3,5-dione is presented in Scheme 1.

Imides or anhydrides obtained in Diels-Alder reaction were used as starting material. 1,7,8,9-Tetramethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione was obtained in reaction of 1,2,3,4-tetramethylcyclopentadiene with furan (30), 1,7,8,9,10-pentamethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione and 1-isopropyl-7-methyl-4-azatricyclo[5.2.2.0²,6]undec-8-ene-3,5-dione is presented in Scheme 1.

Imides or anhydrides obtained in Diels-Alder reaction were used as starting material. 1,7,8,9-Tetramethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione was obtained in reaction of 1,2,3,4-tetramethylcyclopentadiene with furan (30), 1,7,8,9,10-pentamethyl-4-azatricyclo[5.2.1.0²,6]dec-8-ene-3,5-dione and 1-isopropyl-7-methyl-4-azatricyclo[5.2.2.0²,6]undec-8-ene-3,5-dione is presented in Scheme 1.

The obtained tricyclic imides or anhydrides reacted with hydrazine (80% aqueous solution) (21, 30). Next, they were subjected to the reaction with appropriate isothiocyanate in order to be transformed into corresponding thiourea derivatives (Scheme 1). Obtained compounds were purified by flash chromatography. MS, H and ¹³C NMR spectra confirmed the identity of products.

The molecular structure of 4Clc (Fig. 1) was determined by an X-ray crystal structure analysis. The present analysis showed an important feature of this compound, viz. the conformational flexibility. Two molecules cocry stallizing in the solid state adopt different orientations of substituents around the thiourea moiety, both conformers – Z and E – are observed.

Antimicrobial activity

The aim of this study was to compare the type and the position of halogen functionalities at terminal benzene ring of cyclic imide derivatives of thiourea against their antimicrobial activity. For this purpose all obtained compounds were tested in vitro against a number of bacteria, including Gram-positive coccii, Gram-negative rods and fungi.

<table>
<thead>
<tr>
<th>Table 2. Activity of compounds against hospital strains of <em>Staphylococcus aureus</em> (MRSA) and <em>Staphylococcus epidermidis</em> (MRSE) – minimal inhibitory concentrations (MIC, µg/mL).</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2Cl4Fb</strong></td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 53/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 54/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 57/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 79/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 80/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 81/05</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 522/11</td>
</tr>
<tr>
<td><em>S. aureus</em> MRSA 573/11</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 16/04</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 23/04</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 24/04</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 31/04</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 62/05</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 63/05</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 76/05</td>
</tr>
<tr>
<td><em>S. epidermidis</em> MRSE 151/06</td>
</tr>
</tbody>
</table>
The procedure has started with a preliminary screening by the disc diffusion method (24, 25), in order to select derivatives with significant antimicrobial properties. Once detected, the most promising (3Clc, 3Cl4Fb, 3Cl4Fd) were examined for their minimal inhibitory concentration (MIC) by the twofold serial microdilution method (26, 27). The observed data are summarized in Table 1.

According to data generated from this study, almost all compounds presented variable activity against selected Gram-positive bacteria. Compounds 1-(3-chloro-4-fluorophenyl)-3-(1,7,8,9,10-pentamethyl-3,5-dioxo-4-azatricyclo[5.2.1.02,6]dec-8-en-4-yl)thiourea (3Cl4Fb) and 1-(3-chloro-4-fluorophenyl)-3-(1-isopropyl-7-methyl-3,5-dioxo-4-azatricyclo[5.2.2.02,6]-undec-8-en-4-yl)thiourea (3Cl4Fd) were found to be potent against clinically important strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Their inhibitory effect expressed as MIC ranged from 8 to 32 µg/mL. Interestingly, the observed values for 3Cl4Fb and 3Cl4Fd are higher than that reported for their bromophenyl analogues (7), due to the presence of two electron withdrawing elements (F, Cl) attached to the benzene ring. However, only for 2Clb and 3Clc the activity against fungi were observed. The latter may be explained as dependent on the differences between structures of bacterial and fungal cells (34, 35). Remaining compounds of the synthesized class have presented low antimicrobial activity.

The next step was to evaluate the activity of previously assigned most active antibacterial thioureas against hospital methicillin-resistant strains of *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* (MRSE). Observed results have confirmed activity of 3-chloro-4-fluorophenyl derivatives, for which MIC values varied from 32 to 64 µg/mL (Table 2).

One could conclude that for monosubstituted derivatives of cyclic imides the introduction of the smallest molar mass and more electronegative halogen (fluorine and chlorine instead of bromine (7) has decreased the activity. However, disubstituted chlorofluorophenyl thiourea connections of 1,7,8,9-tetramethyl-4-azatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (3Cl4Fa) and 1-isopropyl-7-methyl-3,5-dioxo-4-azatricyclo[5.2.2.02,6]-undec-8-ene-3,5-dione were the most active derivatives presented in this study. It led to the conclusion that also structural isomerism of an imide is an important factor determining the antibacterial potency. Biologically potent 3Cl4Fb and 3Cl4Fd are constitutional isomers of inactive 3Cl4Fc. The activity of 1,7,8,9-tetramethyl-4-azatricyclo[5.2.1.02,6]dec-8-ene-3,5-dione (3Cl4Fa), that is their homolog, was low. To sum up our investigation concerning the activity of cyclic imide derivatives, we confirmed that 3-bromophenyl (7) and 3-chloro-4-fluorophenyl derivatives are the most versatile antibacterial agents.

### Anti-HIV-1 and anti-proliferative activity

The title compounds were tested in the cell-based assay against the human immunodeficiency virus (HIV-1). According to the results, the compounds had a significant antiviral activity. The most active compounds were 3Cl4Fb and 3Cl4Fd, with IC50 values of 29.0 and 16.3 µM, respectively. The data are summarized in Table 3.

#### Table 3. Cytotoxicity and anti-HIV-1 activity.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MT-4 CC50 (µM)</th>
<th>MT-4 EC50 (µM)</th>
<th>HIV-1 IC50 (µM)</th>
<th>HIV-1 EC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2Cla</td>
<td>49.0</td>
<td>&gt;49.0</td>
<td>29.0</td>
<td>&gt;29.0</td>
</tr>
<tr>
<td>3Cla</td>
<td>29.0</td>
<td>&gt;29.0</td>
<td>34.0</td>
<td>&gt;34.0</td>
</tr>
<tr>
<td>4Cla</td>
<td>42.0</td>
<td>&gt;42.0</td>
<td>43.8</td>
<td>&gt;43.8</td>
</tr>
<tr>
<td>3Clb</td>
<td>38.0</td>
<td>&gt;38.0</td>
<td>57.5</td>
<td>&gt;57.5</td>
</tr>
<tr>
<td>3Clc</td>
<td>40.0</td>
<td>&gt;40.0</td>
<td>57.7</td>
<td>&gt;57.7</td>
</tr>
<tr>
<td>4Clb</td>
<td>46.0</td>
<td>&gt;46.0</td>
<td>5.5</td>
<td>&gt;5.5</td>
</tr>
<tr>
<td>2Cla</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>33.5</td>
<td>&gt;33.5</td>
</tr>
<tr>
<td>3Fa</td>
<td>48.6</td>
<td>&gt;48.6</td>
<td>41.5</td>
<td>&gt;41.5</td>
</tr>
<tr>
<td>4Fa</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>38.0</td>
<td>&gt;38.0</td>
</tr>
<tr>
<td>2Fc</td>
<td>46.0</td>
<td>&gt;46.0</td>
<td>37.6</td>
<td>&gt;37.6</td>
</tr>
<tr>
<td>3Fc</td>
<td>33.5</td>
<td>&gt;33.5</td>
<td>46.7</td>
<td>&gt;44.7</td>
</tr>
<tr>
<td>4Fc</td>
<td>51.0</td>
<td>&gt;51.0</td>
<td>43.4</td>
<td>&gt;43.4</td>
</tr>
<tr>
<td>3Cl4Fa</td>
<td>38.0</td>
<td>&gt;38.0</td>
<td>16.3</td>
<td>&gt;16.3</td>
</tr>
<tr>
<td>3Cl4Fb</td>
<td>29.0</td>
<td>&gt;29.0</td>
<td>31.5</td>
<td>&gt;31.5</td>
</tr>
<tr>
<td>3Cl4Fc</td>
<td>16.3</td>
<td>&gt;16.3</td>
<td>40.0</td>
<td>&gt;40.0</td>
</tr>
<tr>
<td>EFVc</td>
<td>0.002</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. Compound concentration (µM) required to reduce the viability of mock-infected MT-4 cells by 50%, as determined by the MTT method. "Compound concentration (µM) required to achieve 50% protection of MT-4 cells from the HIV-1 induced cytopathogenicity, as determined by the MTT method. " Efavirenz.
virus type-1 (HIV-1), using efavirenz as reference inhibitor. The cytotoxicity against the MT4 cells was evaluated in parallel with the antiviral activity (Table 3).

None of tested compounds showed anti-HIV-1 activity, while resulted weakly cytotoxic against MT-4 cells. Only compound 4Cld turned out cytotoxic in a low micromolar range (CC$_{50}$ = 5.5 µM). The higher cytotoxicity of 4Cld prompted us to evaluate its cytotoxicity also for a panel of human cells derived from both hematological and solid tumors. Camptotecin was used as reference compound. As shown in Table 4, it showed cytotoxicity against all tested human leukemia/lymphoma and solid tumor derived cell lines. CC$_{50}$ values comparable to that obtained with MT-4 cells, offering interesting indications for SAR studies, with the aim to design and develop more potent derivatives.

**REFERENCES**


Table 4. Cytotoxicity of 4Cld against human leukemia/lymphoma (CCRF-CEM, WIL-2NS, CCRF-SB) and solid tumor (SK-MEL28, DU145) cell lines.

<table>
<thead>
<tr>
<th></th>
<th>CCRF-CEM</th>
<th>WIL-2NS</th>
<th>CCRF-SB</th>
<th>SK-MEL28</th>
<th>DU145</th>
</tr>
</thead>
<tbody>
<tr>
<td>4Cld CC$_{50}$</td>
<td>8.0</td>
<td>8.4</td>
<td>10.6</td>
<td>11</td>
<td>6.7</td>
</tr>
<tr>
<td>Camptotecin CC$_{50}$</td>
<td>0.003</td>
<td>0.006</td>
<td>0.003</td>
<td>1.0</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Data represent mean values for three independent determinations. Variation among duplicate samples was less than 15%. Compound concentration (µM) required to reduce cell proliferation by 50%, as determined by the MTT method, under conditions allowing untreated controls to undergo at least three consecutive rounds of multiplication. 'CD4' human acute T-lymphoblastic leukemia. 'Human splenic B-lymphoblastoid cells. 'Human acute B-lymphoblastic leukemia. 'Human skin melanoma. 'Human prostate carcinoma.'


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SYNTHESIS AND ANTIMICROBIAL PROPERTIES OF NEW MANDELATE IONIC LIQUIDS

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Abstract: Novel mandelate ionic liquids with quaternary ammonium cations were synthesized and characterized. The compounds exhibit antimicrobial activity and the most potent one is of similar efficacy against Gram+ bacteria as its counterpart chloride. On the other hand, the mandelates are much less active against Gram- bacteria and fungi. QSAR models suggest that, with respect to cation, their potency depends on lipophilicity. The synthesized ionic liquids are also quite cytotoxic against mammalian cells.

Keywords: benzalkonium salts, mandelates, ionic liquids, antimicrobial activity, QSAR

Ionic liquids (ILs), although known since 1914, only in recent years enjoy an enormous growth of interest from chemists of various fields, including electrochemistry, catalysis, analytics, physical chemistry, engineering and industrial chemistry as well as medicinal chemistry (1-9). This is reflected in a steeper than exponential increase in the number of publications and patents every year in recent two decades (10). The reason for this interest is in their unique properties and high tunability, allowing researchers to tailor an IL exactly to their needs with regard to solubility, chirality, catalytic or solving capabilities (and many others) at a relatively low cost.

In pharmaceutical research, apart from typical synthetic and analytical applications, considerable attention is devoted to a possible use of ILs for: (a) overcoming solubility (solvents and co-solvents) issues, (b) improved and controlled delivery (novel formulations of biopolymers), (c) designing of polymorphs of active pharmaceutical ingredients (APIs), (d) or avoiding polymorphism issues by liquid APIs, and finally (e) biologically active ‘Drug Ionic Liquids’ with dual activity (11-14).

Our group investigates biologically active ionic liquids with quaternary ammonium cations (quats) and organic anions in quest for novel antimicrobial compounds with improved activity and pharmaceutical properties.

Quats constitute a recognized group of antibacterials and fungicides. They are widely used as components of home, industrial and medicinal disinfectants, as well as of common antiseptics. The most popular of them include: benzalkonium, benzethonium, cetalkonium, cetlypyridinium, didecyldimethylammonium and domiphene chlorides or bromides.

Quaternary alkylammonium halides are very good precursors for ionic liquids. A simple exchange of an inorganic anion for an organic one allowed us to derive a new class of ILs with biocidal activity comparable to - or even better than - that of the starting ammonium chloride. The synthesized compounds included saccharinate, acesulfamate, L-lactate and L-prolinate anions (15-17).

Another anion investigated was mandelate, and here we have previously prepared very potent mandelate ionic liquids with domiphene, didecyl-
ammonium and mixture of benzalkonium cations (17). The rationale behind design and synthesis of mandelate ILs was that the mandelic acid itself is active against some microorganisms and is used in medicine as a mild antibacterial in urinary tract infections (18), and in cosmetology as an ingredient of chemical face peels (19). Following the path paved in our previous study, we prepared a set of new mandelate ionic liquids and report here their synthesis, structural studies, antibacterial properties and cytotoxicity.

EXPERIMENTAL

Synthesis

In 250 mL two-necked flask equipped with reflux condenser, thermometer and magnetic stirrer, mandelic acid (1) (7.61 g, 0.05 mol) and water (30 mL) were placed. Next, a solution of potassium hydroxide (2.81 g, 0.05 mol) in 10 mL of water was added. The obtained mixture was heated at 60°C for 2 h. A solution of alkylbenzyldimethylammonium chloride (3a-j) (0.05 mol) in water was added and the obtained mixture was heated at 60°C for 6 h. Next, water was distilled off under reduced pressure and content of flask was dried under 60°C and pressure 1-5 mm Hg. To the dry residue 90 mL of acetone (or methanol) was added and the mixture was cooled for 24 h at about -20°C. A precipitate of potassium chloride was collected by filtration. A filtrate was concentrated and dried under reduced pressure.

The purity of prepared compounds was ≥95%. They contained 0.5-1.2% chlorides and ca. 1% water. (Table 1). The water content was determined by using an Aquastar volumetric Karl-Fisher titration. The chloride content was determined by titration with AgNO₃ (Mohr method).

1H NMR and 13C NMR spectra were recorded on a Varian 200 MHz model spectrometer using CDCl₃ or CD₂Cl₂ as solvent and TMS as an internal standard.

Benzyltrimethylammonium mandelate 4a

Yellow wax, 99% yield. 1H NMR (200 MHz, CD₂Cl₂, δ, ppm): 2.86 (s, 9H), 4.37 (s, 2H), 4.81 (s, 1H), 4.83 (s, 1H), 7.15-7.19 (m, 3H), 7.34-7.41 (m, 7H). 13C NMR (50 MHz, CD₂Cl₂, δ, ppm): 52.8, 69.5, 74.7, 127.2, 127.3, 128.2, 128.4, 129.6, 131.0, 133.4, 143.5, 177.0.

Benzylidimethyl-2-hydroxyethylammonium mandelate 4b

Yellow liquid, yield 99%. 1H NMR (200 MHz, CD₂Cl₂, δ, ppm): 2.86 (s, 6H), 3.32-3.36 (m, 3H), 3.90-3.98 (m, 2H), 4.42 (s, 2H), 4.77 (s, 1H), 5.12 (s, 1H), 7.08-7.26 (m, 3H), 7.33-7.52 (m, 7H). 13C NMR (50 MHz, CD₂Cl₂, δ, ppm): 50.7, 56.1, 66.8, 69.2, 74.9, 127.0, 127.7, 128.2, 129.5, 131.0, 133.5, 144.1, 177.2.

Benzylidimethylhexylammonium mandelate 4c

Yellow liquid, yield 98%. 1H NMR (200 MHz, CDCl₃, δ, ppm): 0.89 (t, 3H, J = 6.5 Hz), 1.20-1.30 (m, 6H), 1.52-1.63 (m, 2H), 2.83 (s, 6H), 3.01-3.1 (m, 2H), 3.37 (s, 1H), 4.42 (s, 2H), 4.86(s, 1H), 7.06-7.23 (m, 3H), 7.31-7.48 (m, 3H), 7.49-7.53 (m, 2H). 13C NMR (50 MHz, CDCl₃, δ, ppm): 14.0, 22.5, 22.7, 26.0, 31.4, 49.5, 63.5, 67.6, 74.8, 126.5, 126.8, 127.4, 127.9, 129.3, 130.7, 133.1, 143.9, 176.6.

Table 1. Properties of the prepared compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Form at room temperature</th>
<th>Molecular mass</th>
<th>Chloride content %</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>yellow wax</td>
<td>301.38</td>
<td>1.19</td>
<td>99</td>
</tr>
<tr>
<td>4b</td>
<td>yellow liquid</td>
<td>331.41</td>
<td>1.08</td>
<td>99</td>
</tr>
<tr>
<td>4c</td>
<td>yellow liquid</td>
<td>371.51</td>
<td>0.60</td>
<td>98</td>
</tr>
<tr>
<td>4d</td>
<td>yellow liquid</td>
<td>399.57</td>
<td>1.42</td>
<td>99</td>
</tr>
<tr>
<td>4e</td>
<td>yellow liquid</td>
<td>427.62</td>
<td>1.31</td>
<td>99</td>
</tr>
<tr>
<td>4f</td>
<td>yellow liquid</td>
<td>455.67</td>
<td>0.67</td>
<td>99</td>
</tr>
<tr>
<td>4g</td>
<td>yellow liquid</td>
<td>483.73</td>
<td>1.26</td>
<td>93</td>
</tr>
<tr>
<td>4h</td>
<td>yellow liquid</td>
<td>511.78</td>
<td>0.51</td>
<td>88</td>
</tr>
<tr>
<td>4i</td>
<td>white solid</td>
<td>539.83</td>
<td>1.27</td>
<td>98</td>
</tr>
<tr>
<td>4j</td>
<td>yellow wax</td>
<td>563.85</td>
<td>0.52</td>
<td>99</td>
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</table>
Synthesis and antimicrobial properties of new mandelate ionic liquids

Benzyldimethyloctylammonium mandelate 4d
Yellow liquid, yield 99%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.89 (t, 3H, J = 6.5 Hz), 1.1-1.40
(m, 10H), 1.42-1.66 (m, 2H), 2.84 (s, 6H), 3.0-3.2
(m, 2H), 4.36 (s, 1H), 4.46 (s, 2H), 4.86 (s, 1H),
7.07-7.24 (m, 3H), 7.30-7.45 (m, 5H), 7.47-7.55 (m,
2H). 13C NMR (50 MHz, CDCl3, δ, ppm ): 14.1,
22.6, 22.6, 26.2, 29.0, 29.1, 31.6, 49.3, 63.4, 67.5,
74.7, 126.4, 126.6, 127.3, 127.7, 129.1, 130.6,
132.9, 143.9, 176.3.
Benzyldimethyldecylammonium mandelate 4e
Yellow liquid, yield 99%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.89 (t, 3H, J = 6.4 Hz), 1.10-1.40
(m, 14 H), 1.45-1.65 (m, 2H), 2.88 (s, 6H), 3.093.17 (m, 2H), 3.38 (s, 1H), 4.52 (s, 2H), 4.87 (s, 1H),
7.10-7.24 (m, 3H), 7.30-7.45 (m, 5H), 7.50-7.56 (m,
2H). 13C NMR (50 MHz, CDCl3, δ, ppm): 14.2, 22.7,
22.8, 26.3, 29.3, 29.5, 29.5, 49.4, 63.5, 67.6, 74.8,
126.4, 126.7, 127.4, 127.8, 129.2, 130.7, 133.1,
144.1, 176.4.
Benzyldimethyldodecylammonium mandelate 4f
Yellow liquid, yield 99%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.88 (t, 3H, J = 6.5 Hz), 1.10-1.37
(m, 18H), 1.46-1.65 (m, 2H), 2.84 (s, 6H), 3.04-3.13
(m, 2H), 4.46 (s, 2H), 4.83 (s, 1H), 4.87 (s, 1H),
7.07-7.24 (m, 3H), 7.30-7.45 (m, 5H), 7.50-7.53 (d,
2H, J = 6.8 Hz). 13C NMR (50 MHz, CDCl3, 50
MHz, δ, ppm): 14.3, 22.9, 26.4, 29.4, 29.5, 29.6,
29.7, 29.8, 32.1, 49.5, 63.6, 67.6, 74.8, 126.6, 126.8,
127.5, 127.9, 129.3, 130.7, 133.1, 143.9, 176.6.
Benzyldimethyltetradecylammonium mandelate
4g
Yellow liquid, yield 93%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.88 (t, 3H, J = 6.3 Hz), 1.1-1.35 (m,
22H), 1.50-1.70 (m, 2H), 2.95 (s, 6H), 3.11-3.19 (m,
2H), 3.37 (s, 1H), 4.56 (s, 2H), 4.86 (s, 1H), 7.107.24 (m, 2H), 7.28-7.49 (m, 2H), 7.33-7.45 (m, 5H),
7.50-7.56 (m, 1H). 13C NMR (50 MHz, CDCl3, δ,
ppm): 14.3, 22.9, 26.4, 29.4, 29.5, 29.6, 29.6, 29.8,
29.8, 29.8, 32.1, 49.6, 63.5, 67.7, 74.7, 126.5, 126.8,
127.5, 127.9, 129.3, 130.7, 133.2, 144.0, 176.5.
Benzyldimethylhexadecylammonium mandelate
4h
Yellow liquid, yield 88%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.88 (t, 3H, J = 6.4 Hz), 1.17-1.35
(m, 26H), 1.45-1.67 (m, 2H), 2.87 (s, 6H), 3.06-3.14
(m, 2H), 4.37 (s, 1H), 4.49 (s, 2H), 4.87 (s, 1H),
7.16-7.2 (m, 3H), 7.33-7.45 (m, 5H), 7.49-7.55 (m,
2H). 13C NMR (50 MHz, CDCl3, δ, ppm): 14.3, 22.8,
26.4, 29.4, 29.5, 29.5, 29.6, 29.7, 29.8, 29.8, 32.0,

707

49.4, 63.5, 67.6, 74.8, 126.5, 126.8, 127.4, 127.8,
129.3, 130.7, 133.1, 144.0, 176.5.
Benzyldimethyloctadecylammonium mandelate
4i
White solid, yield 98%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.88 (t, 3H, J = 6.4 Hz); 1.16-1.34
(m, 30H,), 1.48-1.65 (m, 2H), 2.93 (s, 6H), 3.023.11 (m, 2H), 3.91 (s, 1H) 4.49 (s, 2H), 4.86 (s, 1H),
7.10-7.23 (m, 3H), 7.38-7.51 (m, 7H). 13C NMR (50
MHz, CDCl3, δ, ppm): 14.3, 22.8, 26.4, 29.4, 29.5,
29.6, 29.7, 29.9, 32.1, 49.9, 63.2, 67.5, 74.6, 126.7,
126.8, 127.5, 128.0, 129.3, 130.7, 133.1, 143.4,
176.8.
Benzetonium mandelate 4j
Yellow wax, yield 99%. 1H NMR (200 MHz,
CDCl3, δ, ppm): 0.7 (s, 9H), 1.33 (s, 6H), 1.7 (s,
2H), 2.92 (s, 6H), 3.48-3.60 (m, 2H), 3.70-3.83 (m,
4H), 4.00-4.08 (m, 2H), 4.51 (s, 2H), 4.87 (s, 1H),
5.09 (s, 1H), 6.76 (d, 2H, J = 9.0 Hz), 7.05-7.30 (m,
5H), 7.38-7.44 (m, 5H), 7.51-7.56 (m, 2H). 13C
NMR (50 MHz, CDCl3, δ, ppm): 31.8, 31.9, 32.4,
38.0, 50.2, 57.0, 62.7, 65.2, 66.8, 69.2, 69.8, 74.8,
113.6, 126.5, 126.7, 127.3, 127.4, 127.9, 129.2,
130.7, 133.4, 143.0, 144.0, 156.1, 176.7.
X-ray crystallographic data collection and refinement
Single crystals of 4h appeared to be the only
ones suitable for X-ray data collection. A piece of
single crystal of dimensions: 0.56 ◊ 0.37 ◊ 0.23 mm3
was chosen and mounted on 4-circle KUMA KM-4
CCD diffractometer with κ geometry and Mo-target
X-ray tube (λ = 0.71073 Å). The X-ray diffraction
data collection was done at 100 K. 1872 frames were
collected with a ω-scan width of 1O, exposure time
of 15 s/frame. A total of 79785 reflections was collected.
The structure was solved with Olex2 (20) and
refined using XL (21) using the least-squares
method. Hydrogen atoms were located geometrically taking into account average neutron bond length
values for bonds between H and non-H atoms.
CCDC 992879 entry contains the supplementary crystallographic data for the 4h crystal structure. This data can be obtained free of charge from
the Cambridge Crystallographic Data Centre
(www.ccdc.cam.ac.uk/data_request/cif).
Antimicrobial activity tests
The microorganisms used for testing were:
Micrococcus luteus NCTC 7743, Staphylococcus
aureus NCTC 4163, Staphylococcus epidermidis
ATCC 49134, Enterococcus faecium ATCC 49474,


Moraxella catarrhalis ATCC 25238, Escherichia coli ATCC 25922, Serratia marcescens ATCC 8100, Proteus vulgaris NCTC 4635, Pseudomonas aeruginosa NCTC 6749, Bacillus subtilis ATCC 6633, Candida albicans ATCC 10231 and Rhodothula rubra (Demml 1889, Lodder 1934). Standard strains were supplied by the National Collection of Type Cultures (NCTC), London, the American Type Culture Collection (ATCC), and the Polish Collection of Microorganisms (PCM). Rhodothula rubra was obtained from the Department of Pharmaceutical Bacteriology, University of Medical Sciences, Poznań (Poland).

The antimicrobial activity was determined by

Table 2. Crystal data for 4h.

<table>
<thead>
<tr>
<th>Empirical formula</th>
<th>C33H53NO3</th>
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<tr>
<td>Crystal system</td>
<td>triclinic</td>
</tr>
<tr>
<td>Space group</td>
<td>P-1</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>8.7998(1), 10.2725(2), 35.6336(7)</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
<td>84.598(2), 86.353(2), 74.173(2)</td>
</tr>
<tr>
<td>volume (Å³)</td>
<td>3082.83(9)</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>temperature (K)</td>
<td>100(2)</td>
</tr>
<tr>
<td>μ (mm⁻¹)</td>
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</tr>
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<td>Maximal diffraction angle 2θ (°)</td>
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<td>R int</td>
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<tr>
<td>R1 index [I &gt; 2σ(I)]</td>
<td>0.0585</td>
</tr>
<tr>
<td>wR2 [all data]</td>
<td>0.1319</td>
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</tbody>
</table>

Figure 1. Molecular structure of the 4h crystal (only the non-H atoms shown for clarity). Packing diagram and asymmetric unit. Thermal ellipsoids given at 50% probability. The figure prepared in ORTEP3 for Windows (22).
Scheme 1. Synthesis of mandelate ionic liquids

Table 3. In vitro activity of alkylbenzyldimethylammonium mandelate ionic liquids against Gram + bacteria (mg/L, values recalculated to µmol/L given in italics).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Activity</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>4d (C₈)</td>
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<td>MBC</td>
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<tr>
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<td>1251.4</td>
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<tr>
<td>S. aureus</td>
<td>MIC</td>
<td>8</td>
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<tr>
<td></td>
<td>MBC</td>
<td>20.0</td>
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<td>125</td>
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<tr>
<td></td>
<td></td>
<td>312.8</td>
</tr>
<tr>
<td>S. epidermidis</td>
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<td>MBC</td>
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<tr>
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</tr>
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<td>312.8</td>
</tr>
<tr>
<td>E. faecium</td>
<td>MIC</td>
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<tr>
<td></td>
<td>MBC</td>
<td>&gt; 500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt; 2502.7</td>
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<td>B. subtilis</td>
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<tr>
<td></td>
<td>MBC</td>
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<td></td>
<td></td>
<td>500</td>
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<td>Average</td>
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<td></td>
<td>MBC</td>
<td>(1626.8)</td>
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</table>
Table 4. *In vitro* activity of alkylbenzyldimethylammonium mandelate ionic liquids against Gram- bacteria (mg/L, values recalculated to µmol/L given in italics).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Compound</th>
<th>Activity</th>
<th>4d (C8)</th>
<th>4e (C10)</th>
<th>4f (C12)</th>
<th>4g (C14)</th>
<th>4h (C16)</th>
<th>4i (C18)</th>
<th>3g (B)</th>
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<td>MBC</td>
<td></td>
<td>1251.4</td>
<td>145</td>
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<td>8.3</td>
<td>3.9</td>
<td>29.6</td>
<td>1.7</td>
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<td><strong>E. coli</strong></td>
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<td>292.3</td>
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<td>2.1</td>
<td>7.8</td>
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<td>&gt; 500</td>
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<td>62</td>
<td>16</td>
<td>31</td>
<td>62</td>
<td>125</td>
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<td><strong>P. vulgaris</strong></td>
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<td>&gt; 500</td>
<td>250</td>
<td>31</td>
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<td>8</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
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<td>&gt; 500</td>
<td>250</td>
<td>16</td>
<td>31</td>
<td>62</td>
<td>125</td>
<td>16</td>
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<td><strong>P. aeruginosa</strong></td>
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<td>250</td>
<td>31</td>
<td>8</td>
<td>8</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>MBC</td>
<td></td>
<td>&gt; 500</td>
<td>250</td>
<td>16</td>
<td>31</td>
<td>62</td>
<td>125</td>
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<td><strong>Average</strong></td>
<td>MIC</td>
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<td>(2440.1)</td>
<td>482.2</td>
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<td>67.0</td>
<td>125.8</td>
<td>(978.1)</td>
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Table 5. *In vitro* activity of alkylbenzyldimethylammonium mandelate ionic liquids against fungi (mg/L, values recalculated to µmol/L given in italics).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Activity</th>
<th>Compound</th>
<th>4d (C8)</th>
<th>4e (C10)</th>
<th>4f (C12)</th>
<th>4g (C14)</th>
<th>4h (C16)</th>
<th>4i (C18)</th>
<th>3g (B)</th>
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<td><strong>C. albicans</strong></td>
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<td>4</td>
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<td>16</td>
<td>2</td>
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<tr>
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<td>MFC</td>
<td></td>
<td>&gt; 500</td>
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<td><strong>R. rubra</strong></td>
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<td>64.1</td>
<td>121.1</td>
<td>29.6</td>
<td>53.7</td>
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<td>&gt; 500</td>
<td>584.6</td>
<td>35.1</td>
<td>64.1</td>
<td>121.1</td>
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<td>53.7</td>
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<td>90.9</td>
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<td>53.7</td>
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</table>
Table 6. Given below are the slopes a, b and the intercept c of QSAR equations. The standard deviation of the coefficients given in the parentheses. n is the number of compounds selected. log P₀ - the apex of the parabola. Statistical parameters are denoted r - correlation coefficient, r² - the goodness of fit, s - standard error of estimation, p - p-value, asterisks denote at what confidence level the equation is significant, ns - not significant p > 0.05, * p < 0.05, ** p < 0.01. For bilinear equations, also β non-linear term is reported in form of log β (due to β variation over several powers of ten).

<table>
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<th>Microorganism</th>
<th>Activity</th>
<th>Equation</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>logβ</th>
<th>logP₀</th>
<th>n</th>
<th>r</th>
<th>s</th>
<th>p</th>
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<td>G+ bacteria</td>
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<td>(±1.0871)</td>
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<td>(±0.1437)</td>
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<td>G- bacteria *</td>
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<td>0.074</td>
<td>0.0074</td>
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<td>(±0.1783)</td>
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<td>(±0.8936)</td>
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<td>fungi</td>
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<td>(±0.4969)</td>
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<td>(±0.2733)</td>
<td>(±9.3924)</td>
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<td>0.440</td>
<td>0.1772</td>
<td>ns</td>
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<td>(±2.5982)</td>
<td>(±7.9497)</td>
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</table>
the tube dilution method. A series of dilutions for each tested compound or mixture was prepared in Mueller-Hinton broth (bacteria) or Sabouraud broth (fungi) media. The concentrations were: 0.1, 0.2, 0.5, 1, 2, 4, 8, 16, 31, 62, 125, 250, 500 mg/L.

Bacteria were cultured in Mueller–Hinton broth for 24 h at 37°C and fungi in Sabouraud agar for 48 h at 28°C. A suspension of the microorganisms at a concentration of 10^6 cfu mL (cfu = colony forming units) was prepared from each culture and each dilution of the compound was inoculated in a 1:1 ratio.

Growth of the microorganisms (or its lack) was determined visually after incubation for 24 h at 37°C (bacteria) or for 48 h at 28°C (fungi). The lowest concentration at which there was no visible growth was taken as minimal inhibitory concentration (MIC). In order to determine minimal bactericidal or fungicidal concentration (MBC or MFC), one loopful was cultured on an agar medium with inactivates (0.3% lecithin, 3% polysorbate 80, and 0.1% cysteine L) and incubated for 48 h at 37°C (bacteria) or at 28°C (fungi). The lowest concentration at which there was no visible colony growth was taken as MBC or MFC.

Cytotoxicity tests
Cytotoxicity of the studied compounds was evaluated using the MTT assay (TACS® MTT Cell Proliferation Assays, Trevigen) on the fibroblast cell line BJ (ATCC). The cell line was maintained in Eagle’s 1959 MEM (Biomed-Lublin) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen). It was seeded in 75 cm² tissue culture flasks (Falcon, Heidelberg, Germany) and cells were incubated at 37°C in a humidified atmosphere (95% air/5% CO2).

At the time of the assays, cells cultured for 2 days were harvested by trypsinization and cell viability was evaluated by trypan blue exclusion. Cells were cultured in 96-well plates, each well containing 8000 cells in a total volume of 90 µL. After 2 h, 10 µL of NaCl (control) or 10 µL of a water solution of a studied compound were added to each well. The plates with cells were incubated for 24 h. Then, 10 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT reagent) was added to each well and the plates were incubated. After 4 h, the solution from each well was removed and 100 µL of DMSO was added. The plates were reading in a multisensor microplate reader (Cytogen) at 550 nm. The quantity of product, as measured by optical density, was directly proportional to the number of living cells.

Each experimental condition was assayed in triplicate and all experiments were performed at least three times. Several concentrations were tested (1, 2.5, 5, 7.5, 10 µM and greater if needed).

RESULTS AND DISCUSSION

Chemistry
The compounds proposed for the synthesis varied with respect to the length of the long alkyl chain substituent at the cation quaternary nitrogen. We investigated C1, even saturated, non-branched alkyls from C6 to C18 as well as hydroxyethyl (OH) substituents. Another slightly different cation included was benzethonium (B). In a simple synthesis (Scheme 1) 10 novel compounds were obtained (4a-j). Respective, commercially available alkylbenzyl-dimethylammonium chlorides (3a-j) reacted with potassium mandelate (2) to give corresponding alkylbenzyl-dimethylammonium mandelates with high yields (88-99%) (Table 1). Most of them were liquid at room temperature.

Crystallography
For crystallographic analysis single crystals of 4h suitable for X-ray crystal structure data collection were obtained by recrystallization from acetone. 4h crystallizes in the P1 space group in triclinic crystal system. (Table 2). The asymmetric unit is composed of two quaternary ammonium cations and two mandelate counterions. (Fig. 1). Positive charge at nitrogen atom shortens the closest C-C bonds (1.520(2)/GC5 and 1.516(3)/GC5) as compared to the next bond (1.535(3)/GC5 and 1.526(3)/GC5). Electron density moves slightly towards the positive charge and thus the closer to the nitrogen the shorter a bond is. The polar parts are located nearby and the long alkyl chains are extended and parallel to each other, almost along the Z-axis of the crystal reference axis.

Table 7. Cytotoxicity of some of the studied compounds expressed as IC50 (µmol/L) ± SEM (n = 12).

<table>
<thead>
<tr>
<th></th>
<th>4f (C12)</th>
<th>4g (C14)</th>
<th>4h (C16)</th>
<th>4i (C18)</th>
<th>3g (C14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>10.0 ± 0.1</td>
<td>7.4 ± 0.8</td>
<td>7.8 ± 0.8</td>
<td>10.0 ± 0.1</td>
<td>12.5 ± 0.1</td>
</tr>
</tbody>
</table>
The mandelate anions are placed antiparallel one to another with their carboxylate moieties directed in opposite directions. The mandelate aromatic rings are parallel (with the angle between their planes equal to 18.35°). On the contrary, cation aromatic rings are nearly perpendicular (81.49°) in the asymmetric unit. The carboxylate and hydroxyl groups of mandelate form intramolecular hydrogen bonds. The anion interacts with the cation both electrostatically as well as forming short contacts. The whole three-dimensional structure of the crystal has a layered architecture. A similar crystal structure was reported for hexadeclpyridinium saccharinate that is also a low-temperature ionic liquid (16).

**Antimicrobial activity**

All the prepared compounds were tested *in vitro* against ten bacterial and two fungal strains. The minimal inhibitory concentrations (MIC) and minimal bacteriocidal concentrations (MBC) or minimal fungicidal concentrations (MFC) are presented in Tables 3-5.

Compounds 4a and 4c were inactive against all tested microorganisms while 4b exhibits modest activity in *M. luteus*, *S. aureus* and *M. catarrhalis*. 4g and 4j seem overall the most active. In general, the mandelates are more potent in the case of Gram+ bacteria. *S. marcescens*, *P. vulgaris*, *P. aeruginosa* are significantly less sensible to all tested compounds than other investigated microorganisms.
Activity against fungi is satisfactory and shows some peculiarity with 4h (C16) being more potent than 4i (C14) what is found neither in other microorganisms nor in antifungal activity data of benzalkonium chlorides. The most potent 4g has slightly worse MIC and MBC values against Gram+ bacteria than its counterpart chloride 3g. It is however much less active when Gram- bacteria or fungi are considered.

The antimicrobial activity of the studied series of homologues can be well described by simple QSAR equations relating activities (Act) to lipophilicities. The relationships are non-linear. They might be presented in parabolic \( \log(1/\text{Act}) = a \log P + b \log P^2 + c \) or bilinear form \( \log(1/\text{Act}) = a \log P + b \log(\beta P + 1) + c \). Such QSAR equations were proposed for averaged activities of mandelates 4d-4i against Gram+ and Gram- bacteria as well as against fungi. (Table 6, Chart 1). The lipophilicity was expressed by a theoretical descriptor \( \log P \) calculated by Dragon 6.0 (23). The model building was done in BuildQSAR (24).

It is generally accepted that the main mode of antimicrobial action of benzalkonium chlorides and other compounds including quats lies in perturbing of the lipid bilayer of cytoplasmic membrane and thus promoting cell lysis. As the QSAR models for benzalkonium mandelates activities (Chart 1) are very similar to the ones calculated for benzalkonium chlorides by Hansch and Clayton (25), it is very probable that the antimicrobial activity has the same or very similar pharmacodynamics and kinetics in both cases. With respect to cation, it is mainly dependent on its size and lipophilicity. It is noteworthy that bilinear curves fit better the experimental points than the traditionally used parabolic curves. The same relationship was observed by Kubinyi (26) for antimicrobial activities of benzalkonium chlorides and a number of other compounds.

Benzetonium (4j) and domiphene mandelates (DOM, previously described (17)) exhibited better activity than could be inferred from the parabolic equation, which points to the existence of different or additional mechanisms of action in the case of these compounds.

Cytotoxicity

In order to determine selectivity of the prepared compounds, cytotoxicity tests were run for mandelates 4f-i. The IC_{50} values are given in Table 7. As is the case with antimicrobial properties, the values seem to be related to the cation lipophilicity by a non-linear relationship. Unfortunately, IC_{50} values for the studied compounds are comparable to their averaged MIC/MBC/MFC values, which means that the compounds do not possess high selectivity for microbial cells. The most potent 4g is around 1.6 more cytotoxic than its counterpart chloride 3g. It is worth pointing out that even though mandelic acid itself exhibits no harmful action to mammalian cells at the studied concentrations, its benzalkonium salts do so to extent larger than benzalkonium chlorides.

CONCLUSIONS

In conclusion, 10 novel mandelate salts (7 of which were room-temperature ionic liquids) were synthesized. The compounds exhibit antimicrobial activity, which is - with respect to cation - dependent on lipophilicity. The most potent compound has similar MIC and MBC values against Gram+ bacteria when compared to a reference benzalkonium chloride. The activity is however worse in cases of Gram- bacteria and fungi. The mandelate is also more cytotoxic against mammalian cells. Thus, the presence of an organic anion (even not cytotoxic itself) does not always have to be indifferent to the safety of benzalkonium-based antimicrobial agents.

REFERENCES


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Inflammation is a normal protective response to tissue injury caused by physical trauma, noxious chemicals or microbial agents (1). An uncontrolled and persistent inflammation may act as an etiologic factor for many of chronic illnesses such as cancer, autoimmune diseases, cardiovascular diseases, obesity, and diabetes (2, 3). Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by leukocytes, macrophages, and mast cells. Cyclooxygenase (COX), a crucial enzyme in the inflammatory process, plays a key role as a rate-limiting enzyme in the production of potent pro-inflammatory prostaglandins (PGs) biosynthesis, exists in at least two isoforms, designated as COX-1 and COX-2 (4). The side effects of the currently available anti-inflammatory drugs pose a major problem during their clinical uses (5). Hence, the development of anti-inflammatory drugs with lesser adverse effect is needed to be designed. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural sources for curing various diseases (6). The plant derived drugs are considered to be associated with less adverse effects as compared to synthetic compounds. These are still being used in several countries as alternative medicines. Many plants like roots of *Glycyrrhiza glabra*, L, the seeds of *Trigonella foenum-groecum*, L, and the fruits of *Coriandrum sativum* L are used to treat inflammatory conditions (7). *Berberis calliobotrys* belongs to Berberidaceae, a family of three genera flowering plants commonly called barberry. *Berberis calliobotrys* is widely distributed in Afghanistan and Pakistan. The species

**ANTI-INFLAMMATORY, ANALGESIC AND Antipyretic ACTIVITIES OF THE AQUEOUS METHANOLIC EXTRACT OF *BERBERIS CALLIOBOTRYS* IN ALBINO MICE**

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**Abstract:** The aqueous methanolic extract of stem part of *Berberis calliobotrys* (AMEBC) was evaluated for anti-inflammatory, analgesic and antipyretic activities in albino mice. Anti-inflammatory activity was evaluated by using carrageenan and albumin induced paw edema, while the analgesic effect was assessed by using formalin-induced paw licking and acetic acid induced abdominal writhing in mice. The brewer’s yeast-induced pyrexia model was used for antipyretic investigation. Ibuprofen (40 mg/kg) was used as a standard drug in all the three models. The aqueous methanolic extract at both (250 mg/kg and 500 mg/kg) doses, showed highly significant (p < 0.001) reduction in paw edema induced by carrageenan and albumin. Moreover, the aqueous methanolic extract also highly significantly (p < 0.001) reduced (87%) the formalin-induced paw licking at 500 mg/kg. The highly significant (p < 0.001) reductions (24.48% and 37.9%) was also observed in the number of writhings. Furthermore, aqueous methanolic extract also demonstrated significant (p < 0.001) antipyretic activity against yeast induced pyrexia. The maximum effect was observed in all the three parameters at 500 mg/kg dose. The results suggest a potential benefit of the aqueous methanolic extract of *Berberis calliobotrys* in treating conditions associated with inflammation, pain and fever.

**Keywords:** *Berberis calliobotrys*, anti-inflammatory, analgesic, antipyretic, ibuprofen
include trees, shrubs and perennial herbaceous plants. This is a shrub, 1-2 m tall, glabrous; stems are red-brown to pale-brown. Berberis calliobotrys has folkloric claim to be used in the treatment of jaundice, fever, backache, pharyngitis and relief of intestinal colic by the local people of Chitral Valley, Pakistan (8). The present study was carried out to evaluate the analgesic, anti-inflammatory and antipyretic activity of Berberis calliobotrys to validate its folkloric claim.

MATERIALS AND METHODS

Plant material

Stem part of Berberis calliobotrys, common name chowenj, was collected from hilly areas of Quetta, Balochistan. Plant, authenticated by Dr. Rasool Bux, Taxonomist, Department of Botany, University of Balochistan, was used in this study. The dried and coarsely powdered stem part of B. calliobotrys (1.7 kg) was extracted by method of cold maceration by adding 9 L (70 : 30, v/v ratio of methanol to distilled water) solvent. The powder was soaked for 72 h at room temperature with occasional shaking. Then, it was filtered through muslin cloth and Whatman qualitative Grade 1 filter paper. The final filtrate was evaporated with the help of rotary evaporator at 50°C and then, lyophilized to give a yield of 4.8% of extract. For administration, the extract was dissolved in distilled water (9, 10).

Animals used

Albino mice of either sex weighing 20-30 g were used in this study. The animals were housed in propylene cages under controlled conditions of 24 ± 1°C and 12 h light – 12 h dark cycles and ventilation. Standard feed and water were provided ad libitum unless otherwise specified. All animals were treated according to the standard procedures guided by National Research Council (11).

Anti-inflammatory activity

Carrageenan induced paw edema in mice

Anti inflammatory activity of aqueous methanolic extracts in the dose of 250 and 500 mg/kg was evaluated against carrageenan induced paw edema model in mice. Twenty animals were divided into four groups consisting of five animals each. Group I served as control group and received distilled water (2 mL/kg p.o.). Groups II and III received orally aqueous methanolic extract of Berberis calliobotrys at 250 and 500 mg/kg, respectively. Group IV received standard drug ibuprofen at a dose of 40 mg/kg p.o. The linear diameter of the injected paw was then measured at 0, 30, 60, 90 and 120 min of the administration of phlogistic agent, using the Vernier calipers. The increases in the right hind paw diameters induced by the injections of fresh egg albumin were compared with those of the contra-lateral, non-injected left hind paw diameters. The percentage inhibition was calculated by the above mentioned formula (1, 15).

Egg albumin-induced paw edema in mice

Adopting the method prescribed by Muko and Ohiri (14), the paw edema was induced by subplantar administration of 0.1 mL of fresh egg albumin into the right hind paw of mice. Twenty animals were divided into four groups consisting of five animals each. Group I served as control group and received distilled water (2 mL/kg p.o.). Group II and III received orally aqueous methanolic extract of Berberis calliobotrys at 250 and 500 mg/kg, respectively. Group IV received standard drug ibuprofen at a dose of 40 mg/kg p.o. The linear diameter of the injected paw was then measured at 0, 30, 60, 90 and 120 min of the administration of phlogistic agent, using the Vernier calipers. The increases in the right hind paw diameters induced by the injections of fresh egg albumin were compared with those of the contra-lateral, non-injected left hind paw diameters. The percentage inhibition was calculated by the above mentioned formula (1, 15).

Analgesic activity

Acetic acid induced writhing in mice

Analgesic activity of aqueous methanolic extract of Berberis calliobotrys against acetic acid induced writhing (0.6%, v/v in saline, 10 mL/kg, i.p.) in doses of 250 and 500 mg/kg p.o. was conducted in mice (16). The various groups of the animals were orally administered with the extracts (250 and 500 mg/kg b.w.), ibuprofen (40 mg/kg b.w.) and distilled water. After 60 min of treatment, mice were administered with acetic acid (0.6%, v/v in normal saline, 10 mL/kg, i.p.). The number of writhings (characterized by contraction of the abdominal musculature and extension of the hind limbs) exhibited by each animal, started 5 min after acetic acid injection and was counted for 10 min.

Formalin induced paw licking in mice

The procedure described by Bhalke (17), was used for the determination of response to pain
induced by formalin. Twenty µL of a 2.5% solution of formalin (0.9% formaldehyde) was injected subcutaneously under the surface of the left hind paw of each mice. The times spent in licking and biting responses of the injected paw, indicative of pain, was recorded for each animal. The animals were pre-treated with distilled water (10 mL/kg body weight p.o.), ibuprofen (40 mg/kg body weight p.o.) and extracts (250 & 500 mg/kg body weight p.o.), 60 min before the administration of formalin. The responses of mice were observed for the first 5 min (first phase) and then 15–30 min (second phase) post-formalin injection. The % inhibition is calculated by the following formula.

\[
\% \text{ Inhibition} = \frac{\text{Reaction time (control)} - \text{Reaction time (treated)}}{\text{Reaction time (control)}} \times 100
\]  

(3)

Anti-pyretic activity

Yeast induced pyrexia in mice

Albino mice either male or female were divided into four groups of five mice each. The animals were fasted 24 h before the commencement of the experiment but deprived of water only during experiment. Initial rectal temperatures were recorded using a digital thermometer. The pyrexia was induced by subcutaneously injecting 20% (w/v) brewer’s yeast suspension (10 mL/kg) into the mouse’s dorsum region. When the temperature was at peak, 18 h after yeast injection, only mice that showed an increase in temperature of at least 0.7°C were used for the experiment. Group I (control group) was treated with distilled water (10 mL/kg) p.o. Groups II and III received AMEBC in doses of 250 and 500 mg/kg, p.o., respectively. Group IV was treated with standard drug - ibuprofen 40 mg/kg, p.o. The rectal temperature was measured at 0, 1, 2, 3 and 4 h after each agent administration (18).

Statistical analysis

The results are expressed as the means ± SEM. Parametric data were compared to control group and were assessed by the method of one-way ANOVA and two-way ANOVA followed by Bonferroni post-hoc tests. Values p < 0.05 were considered as statistically significant.

RESULTS

Aqueous methanolic extract of Berberis calliobotrys significantly (p < 0.001) reduced the edema formation of the paw induced by carrageenan at all assessment times. At 0 min, aqueous methanolic extract at dose of 250 mg/kg showed non-significant effect but aqueous methanolic extract at a dose of 500 mg/kg showed significant (p < 0.01) effect. At 30 min, aqueous methanolic extract at both doses, showed highly significant (p < 0.001) reduction in paw edema, that showed its potent anti-inflammatory effect. This result demonstrated dose-time related significant reduction by aqueous methanolic extract. Ibuprofen (40 mg/kg), similarly produced significant inhibitory effect of the paw edema as compared to normal control group (Table 1).

Table 1. Effect of aqueous methanolic extract of Berberis calliobotrys at 250 and 500 mg/kg doses against carrageenan induced paw edema in mice.

<table>
<thead>
<tr>
<th>Treatment (dose, mg/kg)</th>
<th>Inflammation induced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 0 min (mm) (%) inhibition</td>
</tr>
<tr>
<td>Control (2 mL/kg)</td>
<td>3.46 ± 0.051</td>
</tr>
<tr>
<td>Aqueous methanolic extract (250 mg/kg)</td>
<td>3.02 ± 0.086** (12.7%)</td>
</tr>
<tr>
<td>Aqueous methanolic extract (500 mg/kg)</td>
<td>2.8 ± 0.12** (19.0%)</td>
</tr>
<tr>
<td>Ibuprofen (40 mg/kg)</td>
<td>2.9 ± 0.025%* (16.1%)</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 5). Key: *** = (p < 0.001) highly significant, ** = (p < 0.01) * = (p < 0.5) and n.s = non-significant as compared to control.
1). The aqueous methanolic extract (250 and 500 mg/kg) significantly (p < 0.001) reduced the edema formation of the paw induced by fresh albumin injection from 30 min to onward at all assessment times. The maximum inhibition was observed at 120 min. Ibuprofen at the dose of 40 mg/kg produced time-related significant (p < 0.05-0.001) inhibitory effect of the paw edema as compared to normal control group (Table 2).

The animals which were pretreated with aqueous methanolic extract at the doses of 250 and 500 mg/kg showed significant reductions (p < 0.001) in the number of writhings with % inhibition of 24.48 and 37.9%, respectively. Whereas intra peritoneal injection of acetic acid to the control group elicited (72.5 ± 1.19) writhings counted in 10 min. Ibuprofen at a dose of 40 mg/kg orally showed a marked inhibition (68.5 %) of writhings (Table 3).

Aqueous methanolic extract produced a significant (p < 0.001) dose-dependent inhibition of nociceptive reaction induced by formalin with peak effect (87% inhibition) produced at the highest dose of 500 mg/kg. Ibuprofen at the dose of 40 mg/kg produced marked inhibition (89%). In the second phase, the total duration of nociceptive reaction in the control group was 203.0 ± 12.3 s. The effect of aqueous methanolic extract in inhibiting the biting and licking response was also significant (p < 0.001). Ibuprofen at the dose of 40 mg/kg produced marked inhibition (92%) of formalin induced paw licking (Table 4).

Table 2. Effect of aqueous methanolic extract of *Berberis calliobotrys* at 250 and 500 mg/kg against egg albumin induced paw edema in mice.

<table>
<thead>
<tr>
<th>Treatment (dose, mg/kg)</th>
<th>Inflammation induced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 0 min (mm) (% inhibition)</td>
</tr>
<tr>
<td>Control (2 mL/kg)</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>Aqueous methanolic extract (250 mg/kg)</td>
<td>3.38 ± 0.15 (6.1%)</td>
</tr>
<tr>
<td>Aqueous methanolic extract (500 mg/kg)</td>
<td>3.14 ± 0.09 (12.7%)</td>
</tr>
<tr>
<td>Ibuprofen (40 mg/kg)</td>
<td>3.0 ± 0.12 (16.6%)</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 5). Key: *** = (p < 0.001) highly significant, ** = (p < 0.01) * = (p < 0.5) and n.s = non-significant as compared to control.

Table 3. Effect of aqueous methanolic extract of *Berberis calliobotrys* against acetic acid induced writhing in mice.

<table>
<thead>
<tr>
<th>Treatment (Dose, mg/kg)</th>
<th>No. of writhings</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (10 mL/kg)</td>
<td>72.5 ± 1.19</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous methanolic extract (250 mg/kg)</td>
<td>54.75 ± 0.854 ***</td>
<td>24.48%</td>
</tr>
<tr>
<td>Aqueous methanolic extract (500 mg/kg)</td>
<td>45 ± 2.345 ***</td>
<td>37.90%</td>
</tr>
<tr>
<td>Ibuprofen (40 mg/kg)</td>
<td>22.83 ± 1.14 ***</td>
<td>68.50%</td>
</tr>
</tbody>
</table>

Results are expressed as the means ± SEM (n = 5). Key: *** = (p < 0.001) highly significant as compared to control.
The *Berberis calliobotrys* extract (500 mg/kg) produced the highly significant (p < 0.001) inhibition of pyrexia after induction. Whereas, 250 mg/kg of extract produced significant (p < 0.01) inhibition of pyrexia after 1 h of induction of pyrexia but becomes less significant (p < 0.05) after 3 h of post induction (Table 5).

**DISCUSSION**

The anti-inflammatory activity of *Berberis calliobotrys* was studied by using carrageenan and albumin induced paw inflammation. The aqueous methanolic extract exhibited significant anti-inflammatory activity. Carrageenan-induced edema falls in the category of acute inflammation, which involves the synthesis or release of inflammatory mediators at the injured site which further cause pain and fever. The aqueous methanolic extract may produce its anti-inflammatory effect by blocking the release of these inflammatory mediators. Our study agrees with the previous study conducted by Bhalke (17).

The result of this study suggests that aqueous methanolic extract of *Berberis calliobotrys* has anti-inflammatory effect comparable to those of the standard drug, ibuprofen. Carrageenan-induced inflam-
mal inflammatory process is believed to be biphasic. The initial phase seen at first hour is attributed to the release of histamine and serotonin. The second accelerating phase of swelling is due to release of prostaglandins (PGs), bradykinin and lysozyme. It has been reported that the second phase of inflammation is sensitive to both clinically useful steroidal and non-steroidal anti-inflammatory agents (19). Aqueous methanolic extract produced significant (p < 0.001) inhibition of paw edema by carrageenan injection in mice after 30 min and continued during all phases of inflammation in both groups. This activity may be assumed probably due to the inhibition of different aspects and chemical mediators of inflammation as established for ibuprofen.

The anti-inflammatory activity of aqueous methanolic extract was also evaluated by using egg albumin induced paw edema in mice. The aqueous methanolic extract significantly (p < 0.001) reduced inflammation in 250 and 500 mg/kg doses. It indicates that aqueous methanolic extract may inhibit inflammation by blocking the release of histamine and 5-HT, which are released by egg albumin. Histamine is an important mediator of inflammation, a potent vasodilating substance and is also involved in increasing vascular permeability (20). The edema produced by egg albumin was effectively suppressed by the aqueous methanolic extract in both doses. The present experimental findings tend to suggest that the extract might demonstrate anti-inflammatory actions by inhibiting the synthesis, release or action of inflammatory mediators such as histamine, serotonin, cytokines and prostaglandins (21).

Furthermore, the abdominal constriction response induced by acetic acid is a sensitive process to evaluate peripherally acting analgesics (22). Acetic acid has been reported to cause hyperalgesia by liberating endogenous substances such as prostaglandins, leukotrienes, 5-HT, histamine and kinins, which have been implicated in the mediation of pain perception. The dose-dependent inhibition of writhings induced by acetic acid in this study suggests a peripherally mediated analgesic activity based on the association of the model with stimulation of peripheral receptors especially the local peritoneal receptors at the surface of cells lining the peritoneal cavity (23, 24). The aqueous methanolic extract in 250 and 500 mg/kg doses produced highly significant (p < 0.001) reduction in abdominal constrictions and stretching jerk of the hind limb. The significant reduction in acetic acid-induced writhes by aqueous methanolic extract at the dose of 250 and 500 mg/kg suggests their analgesic effect may be peripherally mediated via the inhibition of synthesis and release of PGs and other endogenous substances as already proposed by Koster (25).

For analgesic activity, the formalin-induced paw licking test was conducted. This test possesses two distinctive phases: first phase measures direct chemical stimulation of nociceptors (neurogenic phase), whereas second phase is dependent on peripheral inflammation and central processing (inflammatory phase). The early phase is due to a direct effect on nociceptors and a consequent C-fibre activation, whereas second phase involve histamine, serotonin, PGs, NO and bradykinin (26). Thus, both centrally and peripherally mediated actions can be measured. An immediate and intense increase in impulsive activity of C afferent fiber, caused by formalin injection, evokes a diverse quantifiable behavior indicative of pain demonstrated as paw licking by the animals (27). The aqueous methanolic extract in 250 and 500 mg/kg doses produced highly significant (p < 0.001) reduction in paw licking in mice indicating that its activity may be because of its central as well as peripheral action as previously reported by Begum (28) and Ghannadi (29).

The yeast-induced hyperthermia in mice was employed to investigate the antipyretic activity of extract in 250 and 500 mg/kg doses. It was found that yeast extract caused a significant reduction in rectal temperature in a dose dependent manner. The yeast induces pathogenic fever by enhancing the production of prostaglandins, mainly PGE2, which elevates the set point of the thermoregulatory centre in hypothalamus (30). The hypothalamus regulates body temperature with a fragile balance between heat production and heat loss through the set-point control (31). In this approach, the body matches the brain blood temperature with the new set point made by the hypothalamus. The infected or damaged tissue initiates the improved formation of pro-inflammatory mediators (cytokines like interleukins and TNF-α), which increase the synthesis of prostaglandin E2 near the pre-optic hypothalamic area, thereby triggering the hypothalamus to raise body temperature (32). Antipyretic activity is commonly stated as characteristic of drugs or compounds which have an inhibitory effect on prostaglandin-biosynthesis (33). The possible mechanism of antipyretic activity of AMEBC might be through inhibition of prostaglandin E2, concentration especially in hypothalamus by its action on newly reported isoenzyme cyclooxygenase 3 (COX 3), present in cerebral cortex and some other tissues that has possible role in pyrexia (34). It has been previously studied that
COX 3 is inhibited by acetaminophen and some other NSAIDs.

CONCLUSIONS

It is conceivable from this study that aqueous methanolic extract of Berberis calliobotrys exhibited analgesic, anti-inflammatory and antipyretic effects through different mechanisms. It also validates the traditional use of this plant in the treatment of various types of pain, fever and inflammation. However, further studies are required to isolate active compounds from the potent extracts and to elucidate their exact mechanism of action.

REFERENCES


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Polish Pharmaceutical Society

Atorvastatin - \((3\,R,5\,R)\)-7-[2-(4-fluorophenyl)-3-phenyl-4-(phenylcarbamoyl)-5-propan-2-yl pyrrol-1-yl]-3,5-dihydroxyheptanoic acid (Fig. 1) is a competitive inhibitor of 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA) which is an early rate limiting step in cholesterol biosynthesis (1). This inhibition leads to consumption of intracellular cholesterol, which increases the expression of low density lipoprotein (LDL) receptor on hepatocytes resulting in a fall in serum LDL cholesterol concentration to about 40% and high systemic disappearance of LDL cholesterol (1, 2). Statins also cause small reduction in triglyceride levels by 10-20% and increase high density lipoprotein reducing the risks of morbidity and mortality due to cardiovascular events (1). Strokes and coronary heart diseases are major cardiovascular events that lead to death (3). Hypercholesterolemia has been classified as major factor in the development of atherosclerosis and coronary heart disease (3). Currently, atorvastatin is used for the treatment of hypercholesterolemia (1).

Pharmaceutical quality assessments were satisfactory and within limits for all atorvastatin tested products. The average weight ranged from 206.6 ± 8.40 to 330 ± 3.92 mg and the %RSDs were within the permitted limits as per USP. Tablet hardness ranged from 102 ± 1.41 to 197.4 ± 6.88 kg and drug contents ranged from 92.2% to 105.3%. All products disintegrated within permitted time limits and showed very rapid dissolution. Products released more than 85% of their drug contents in less than 15 min. Our results showed that all tested innovator and generic atorvastatin products were of good pharmaceutical quality. Despite the lack of in vivo evaluation, our results indicate that these products are equivalent in vitro. Considering the in vitro release characteristics, these products might be used interchangeably. However, regulatory authorities permit the use of in vitro data in establishing similarity between immediate release oral dosage forms containing biopharmaceutical classification system class I and III drugs only.

**Keywords:** atorvastatin, pharmaceutical quality, bioequivalence, dissolution

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**Figure 1. Chemical structure of atorvastatin**

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phate buffer of pH 7.4 (4). Such data suggest extremely poor dissolution in gastrointestinal tract, especially in the fasted stomach (4). According to the biopharmaceutical classification system (BCS), atorvastatin is a BCS class II (low solubility and high permeability) compound (5). Atorvastatin is rapidly absorbed in upper gastrointestinal tract. Its oral bioavailability is about 14% (4, 6). This low bioavailability might be due to poor dissolution, presystemic clearance in the gut wall and first pass effect (3). Atorvastatin is acid sensitive drug and accordingly the tablet formulation includes calcium carbonate (CaCO₃) in order to maintain alkaline environment around the active pharmaceutical ingredient (API) (3). The maximum plasma concentration of atorvastatin can be obtained within 2-3 h with long half-life of 20 h (7).

Drugs with poor water solubility often show unpredictable intestinal absorption and high intra- and inter-subject variability (8). Therefore, it is necessary for the health regulatory authorities, manufacturers and researchers to keep a continuous surveillance of poorly water-soluble drugs available on the markets to ensure the accessibility of high quality medicines (9). In 2010, the innovator atorvastatin was the top-selling prescription medication in the United States and generated more than $7 billion in total revenue (10). In June 2011, the innovator’s patent expired and since then many generic versions entered the pharmaceutical markets. Despite the attempts to globalize the principles of the World Trade Organization’s Trade Related Aspects of Intellectual Property Rights (WTO-TRIPS), still the laws in many countries do not support patent protection including pharmaceuticals (11, 12). Many generic versions of innovator products existed before the expiration of patent protection. Countries are now given a transition period till 2016 to upgrade their patent protection laws to meet the WTO’s standards.

Generics and innovator products should be equivalents to be used interchangeably. Since 1960s, in vivo bioequivalence studies have emerged as “gold standards” to prove equivalency between a generic and its innovator counterpart (13). Studies showed that the in vivo bioavailability of a drug formulated into an oral dosage form depends on its dissolution and release characteristics (14). Today, dissolution testing is a widely used quality control tool to ensure batch-to-batch consistency and as an in vitro surrogate for in vivo performance (15).

The current study compares the pharmaceutical quality of innovator atorvastatin with its locally available generic versions in Palestine as well as the quality of the drug and to assess the suitability of interchangeability between the innovator and its generics and between generics themselves. All products were assessed against established procedures used to assess the qualitative and quantitative pharmaceutical quality characteristics of atorvastatin tablets.

**EXPERIMENTAL**

**Materials**

Atorvastatin calcium working standard was provided by Beit Jala Pharmaceutical Company, Beit Jala, Palestine. Samples of innovator and all four locally manufactured generic atorvastatin products (20 mg tablet formulations) available on the local market were purchased from a local retail pharmacy shop for testing. Purchased tablets were checked for manufacturing license numbers, batch numbers, production and expiration dates. The qualitative compositions of all products was taken from the products’ summary characteristics and compared against the innovator. The products were coded as AS-1 (innovator), AS-2, AS-3, AS-4 and AS-5. All generic products (AS-2 through AS-5) were compared against the innovator (AS-1). All chemicals and reagents used were of HPLC grade.

**Per tablet price comparison of different atorvastatin products**

The price differences between innovator and generic products were compared using per tablet percent price difference which was calculated according to the following equation:

\[
\text{Percent price difference} = \left( \frac{\text{price of innovator} - \text{price of generic product}}{\text{price of innovator}} \right) \times 100\%
\]

**Testing physicochemical properties and pharmaceutical quality**

Innovator and generic products were evaluated for their organoleptic properties (shape and color), assay, weight uniformity, tablet hardness, disintegration and dissolution. All atorvastatin tablets were visually inspected for shape, color, absence of black spots and/or preched edges.

**Assay**

The amount of atorvastatin calcium in each product was tested according to the United State Pharmacopeia (USP) (16). Twenty tablets from each product were crushed into a very fine powder using mortar and pestle. An equivalent amount of 114 mg of atorvastatin powder was weighed and dissolved in 60 mL of mobile phase (50% acetonitrile and
Pharmaceutical quality of generic atorvastatin products compared with... 727

50% buffer). The mixture was sonicated for about 15 min and the volume was adjusted using mobile phase. The solution was passed through 0.5 µm filter. The first 10 mL of the filtrate was dismissed. The clear filtrate was injected into the HPLC (Dionex, Sunnyvale, CA) with auto-sampler (QCA 150). The amounts of atorvastatin were analyzed against atorvastatin reference standard USP which was injected separately. Samples were passed through the column (Lichrosphere RP18, 125 × 4 mm, 5 µm, Merck, Germany) at 1.5 mL/min.

The amount of atorvastatin calcium was calculated using the following equation:

\[
\text{Weight uniformity} = \frac{\text{Area under the curve (AUC) of sample}}{\text{AUC of reference standard}} \times 100\%
\]

To assess the weight uniformity of atorvastatin tablets, twenty tablets of each innovator and generic products were randomly selected and weighted individually using analytical balance (262 SMA-FR Series, Switzerland). Percentage deviation of each individual tablet from the mean was evaluated according to the USP standard method (16).

**Hardness**

The hardness test was carried out on tablets to determine the resistance of tablet to breakage. The hardness of 5 tablets of atorvastatin products was measured randomly using hardness tester (Erweka, Germany) and the crushing strength was recorded.

**Disintegration**

Evaluation of disintegration time of atorvastatin tablets was done according to the procedure described in the USP (16). One tablet was placed in each of the 6 tubes of the basket of the disintegration apparatus (Erweka, Germany). Each tube was filled with water and the temperature was adjusted at 37°C. Disintegration times were noted. According to the USP, immediate release film coated tablets should disintegrate completely in ≤ 30 min.

**Dissolution**

Dissolution of tested atorvastatin tablets was evaluated using USP paddle 2 dissolution apparatus (Caleva 8ST, Germany). In accordance with the test standards recommended by the FDA, six tablets of each product were tested. In the dissolution apparatus, one tablet was placed in each vessel with a paddle stirrer at 75 rpm filled with 900 mL of dissolution media (0.05 M phosphate buffer, pH 6.8) and the temperature was adjusted at 37 ± 0.5°C. Aliquots of 2 mL from each dissolution vessel were removed after 5, 10, 20, 30, and 45 min. The percents of atorvastatin release were analyzed using HPLC (Dionex, CA).

**RESULTS AND DISCUSSION**

**Price differentials**

All atorvastatin tablets were evaluated for their prices and pharmaceutical quality. Comparing prices, the innovator atorvastatin was more expensive than three out of four generic atorvastatin products (Table 1). All visually inspected atorvastatin tablets were of white color and showed oblong shape. Overall, the tablets were of good quality and did not show any signs of defects with respect to shape, color, presence of black spots or preached edges. Qualitatively, the composition of all tested tablets was similar. In addition to atorvastatin, tablets contained calcium carbonate, candelilla wax, croscarmellose sodium, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose monohydrate, magnesium stearate, microcrystalline cellulose, opadry white Ys-1-7040, polyethylene glycol, polysorbate 80, simethicone emulsion, talc, and titanium dioxide.

<table>
<thead>
<tr>
<th>Product code</th>
<th>Number of tablets in each package</th>
<th>Price of the package (JD)</th>
<th>Per tablet percent price difference with innovator (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-1 (innovator)</td>
<td>30</td>
<td>10.7</td>
<td>-</td>
</tr>
<tr>
<td>AS-2</td>
<td>30</td>
<td>7.3</td>
<td>32.5</td>
</tr>
<tr>
<td>AS-3</td>
<td>28</td>
<td>9.1</td>
<td>9.14</td>
</tr>
<tr>
<td>AS-4</td>
<td>30</td>
<td>9.1</td>
<td>15.7</td>
</tr>
<tr>
<td>AS-5</td>
<td>30</td>
<td>12.7</td>
<td>-18.3</td>
</tr>
</tbody>
</table>

JD = Jordanian Dinar
themselves existed in poor, middle and high income countries. Studies showed that on average, an innovator product is 2.6-folds more expensive than the lowest priced generic version, although the difference may vary by more than 10-fold (17, 18). Our results were in line with these findings, since three out of four generics were less expensive than the innovator product (Table 1).

This is particularly important since a considerable segment of the population in developing countries cannot afford expensive brands and paradoxically patients prefer imported medications as they believe that these products have superior activity and safety profiles compared with the locally manufactured generics (19). Our study showed that one locally manufactured generic was more expensive than the innovator. We do not know if this has implications on the market-share of this product compared to the innovator. Previous reports showed that lack of incentives to physicians, pharmacists and higher prices disfavored the sales of generics (17, 18). Healthcare expenditures are on the rise worldwide and financially constrained systems are under pressure to seek ways to cut costs in a safe and effective manner (10). In the US, switching from the innovator atorvastatin to generics is estimated to save $4.5 billion annually by 2014 (10). Similarly in developing countries, switching to affordable generics can improve access to medicines when needed and can bring significant savings to healthcare systems and general public.

Pharmaceutical quality

All atorvastatin containing products tested were within the ± 10% limit difference set by the USP and passed the weight uniformity test as shown in Table 2. Atorvastatin containing products were tested for their drug contents. All tested products (AS-1 through AS-5) passed the test and variations were within specifications (92-105%). The lowest amount was observed in AS-3 and the highest amount was found in AS-5 (Table 2). Hardness of all tested tablets was in the range of 102-197.4 kg as shown in Table 2. The disintegration time for all brands was within 3 min with an average of 2.5 min. The highest disintegration time (3 min) was observed for AS-4.

All tablet products were subjected to dissolution testing using dissolution media at pH 6.8. The dissolution profiles of the five tested products are shown in Figure 2. All tested products showed very rapid dissolution and released ≥ 85% of their atorvastatin contents in ≤ 15 min. Very rapid dissolving tablets are considered essentially similar without a need for similarity (f2) and difference (f1) factors.

Regulatory authorities require a proof of similarity between a generic and innovator products in order to grant the generic a marketing authorization (13).

Interchangeability between an innovator and its generic versions is based on the proof of similarity provided. Assessing the pharmaceutical quality of locally manufactured generics is particularly important for determining their suitability for interchangeability with innovator products. In this study, we used compendial and non-compendial tests to assess the pharmaceutical quality of innovator and generic versions of atorvastatin. Our results showed that all tested products lack physical defects in shape, color or presence of other flaws. Absence of such flaws is particularly important for consumer acceptability and patient compliance (20). The weight uniformity and assay tests showed that products contain the same amount of atorvastatin which can ensure the dose uniformity and that all products contain the labeled atorvastatin amounts. Hardness testing showed that all products possess sufficient strength to withstand stress without losing any parts of their components of tablets during packaging and handling of these products. Our results showed that the generic product (AS-4) showed higher hardness value compared to the innovator which explained its relatively delayed disintegration compared to the innovator (21). Other products disintegrated in less

<table>
<thead>
<tr>
<th>Product code</th>
<th>Average weight (mg) ± SD; (%RSD)</th>
<th>Hardness (kg) ± SD</th>
<th>Assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-1 (innovator)</td>
<td>206.25 ± 2.84; (1.38)</td>
<td>122 ± 11.19</td>
<td>97.6</td>
</tr>
<tr>
<td>AS-2</td>
<td>206.6 ± 8.40; (3.23)</td>
<td>112 ± 13.63</td>
<td>102.5</td>
</tr>
<tr>
<td>AS-3</td>
<td>315.8 ± 1.22; (0.39)</td>
<td>102 ± 1.41</td>
<td>92.2</td>
</tr>
<tr>
<td>AS-4</td>
<td>306.75 ± 7.18; (2.17)</td>
<td>197.4 ± 6.88</td>
<td>104.2</td>
</tr>
<tr>
<td>AS-5</td>
<td>330 ± 3.92; (1.28)</td>
<td>110.4 ± 21.91</td>
<td>105.3</td>
</tr>
</tbody>
</table>
than 3 min which might suggest similar disintegrant contents. Previous studies showed that disintegration time is particularly important in predicting dissolution and subsequent release of drug contents (21). Dissolution testing has emerged as crucial tool in ensuring the release characteristics and batch to batch variability. The dissolution profile of all generics were tested in simulated intestinal fluid and compared with the innovator. All tablets showed very rapid dissolution and the drug released from the innovator and locally manufactured atorvastatin tablets was greater than 85% in ≤ 15 min. These results predict that all tested products can release their atorvastatin contents in a comparable manner. In fact, Amidon et al. showed that if a drug from two different dosage forms is presented at the intestinal lumen at the same molar concentration, there should be no difference in the absorption and any difference may come from within subject pathophysiological variability (22).

Our results can be interpreted considering the limitation of lacking in vivo investigations. Despite the emergence of the biopharmaceutical classification system and the concept of biowaiver and the wide adaptation by regulatory authorities in the US, Europe and Japan, still, authorities see that in vivo pharmacokinetic bioequivalence studies are pivotal in establishing bioequivalence and interchangeability between oral dosage forms containing atorvastatin. Using the FDA official dissolution method to establish the release profiles of different formulations containing atorvastatin in three pH media of 1.2, 4.5 and 6.8 was another major limitation. In this study, we did not use a biorelevant discriminative dissolution method (23). However, the official FDA method was reportedly used in evaluating the in vitro equivalence of tablets containing atorvastatin (9).

CONCLUSION

In conclusion, our results showed that generally, generic atorvastatin are offered to the consumers at lower cost than innovator. Switching to generic prescription can bring significant cost reduction for the public consumers and the healthcare authorities. This could be important for local health authorities to procure high quality medications at lower costs. Furthermore, our results indicate that locally manufactured generics containing atorvastatin (20 mg) are of good pharmaceutical quality compared to the innovator product. Despite the lack of in vivo evaluation, our results indicate that these products are equivalents in vitro. Considering the in vitro release characteristics, these products might be used interchangeably. However, based on the concept of biowaiver, regulatory authorities permit the use of in vitro data in establishing similarity between immediate release oral dosage forms containing BCS class I and III drugs only.

Competing interests

All authors declare that this study was conducted for the purpose of scientific research only. All authors declare no competing interests.
REFERENCES


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In the twenty-first century pharmaceutical technology the dominating tendency is to seek a drug form which would provide a desired therapeutic effect with a minimum of side effects. Therefore, scientists create drug forms which both provide the correct dose of the medicinal substance to a specific organ and ensure its release for a specified period of time (1–3). In clinical practice the use of sustained release preparations (8–12 h) enables the use of a lower daily dose, which results in smaller variation of the concentration of medicinal substance in the blood. In order to obtain immediate and sustained release, the drug form technology has been enriched with newly developed multi-compartment systems whose structure makes it possible to meet the above-mentioned requirements. Thanks to this universal drug form it is possible to overcome many of the disadvantages arising from the use of conventional drug forms (4). The drug dose is divided into many sub-units – containers, each of which behaves like an individual form, providing medicinal substance in a predetermined place and in a predetermined way. If in the multi-compartment system several medicinal substances of different places of release are used, such a drug form can successfully compete with other forms, either in monotherapy or in combination therapy (5–7).

Multi-compartment system is superior to traditional drug forms in terms of more efficient control of release and absorption place. As a result, a better control of the concentration of medicinal substance in blood or tissues is obtained, leading to improved safety of pharmacotherapy (8–10). Furthermore, this kind of system provides the possibility to model the profile of medicinal substance release and absorption by placing in one capsule e.g., mini tablets of different compositions or of different coatings (5, 11, 12). Control of the rate of medicinal substance release is achieved inter alia through the use of polymer coatings of varied thickness, soluble at different pH values or through the use of excipients, delaying disintegration of the tablet or the release of the medicinal substance (11–13).

One of the main areas where modified release of medicinal substance is often used is the treatment of pain and inflammations. Ketoprofen (the II class
of BCS system) (14) is a representative of the group of non-steroidal anti-inflammatory drugs (NSAID) (15) and it is widely used in the treatment of pain, in cases of inflammation and rheumatic diseases (16). When administered in the drug form of unmodified release, it is rapidly absorbed, achieving the maximum concentration in blood within 0.5 to 2 h (17, 18). After this time, its concentration decreases rapidly to a very low level, thus in the long-term treatment it is necessary to take multiple doses of the drug per day in order to maintain the therapeutic concentration (9).

Multi-compartment systems enable the release of desired amount of active substance in a selected section of the gastrointestinal tract (19, 20), according to a determined dosing schedule. Thanks to them it is often possible to reduce the quantity of drug doses to one per day (21).

The aim of the study was to construct and test a multi-compartmentment system, consisting of mini tablets of ketoprofen, enclosed in a capsule of hypromellose. The mini tablets obtained by the usage of different technologies and having different properties, created multi-compartment systems in suitable configurations. These systems allowed to change the composition and the dose of medicinal substances easily. It was also possible to modify the release profiles of applied substance.

MATERIALS AND METHODS

In the first stage of the study, using a tablet press (EK0 - Korsch) with a stamp having a diameter of 5 mm, two types of mini tablets with ketoprofen (Lee Pharma) as a model substance were prepared: immediate release mini tablets (IR) of a weight of 50 mg and the medicinal substance content of 12.5 mg as well as sustained release mini tablets (SR) of a weight of 60 mg and ketoprofen content of 12.5 mg. Both kinds of mini tablets were prepared using direct compression. Part of the immediate release mini-tablets were coated with enteric coating (ACRYL- EZE PINK, Colorcon), which resulted in mini tablets releasing drug substance with a delay (IRc). Mini tablets/cores were coated according to the Würster method (UNIGLATT, Glatt) (22). Weight gain of the tablets in the coating process was about 16%. The composition of the tablet mass of IR and SR mini tablets and its properties are shown in Table 1.

Release profiles were tested for each type of mini tablets, pursuant to the EMA (European Medicines Agency) guidelines, in three media: 0.1 M HCl, phosphate buffer pH 4.5 and phosphate buffer pH 6.8. In addition, release test in the phosphate buffer pH 6.8 over the course of 12 h was carried out for SR tablets. Concentration of ketoprofen in the tested samples was determined spectrophotometrically (spectrophotometer UV 300 UV:Visible, Unicam) at a wavelength λ = 259 nm. The correlation coefficients for standard curves drawn for ketoprofen in particular media were respectively:

- 0.1 M HCl (r² = 0.9995)
- phosphate buffer, pH 4.5 (r² = 0.9979)
- phosphate buffer, pH 6.8 (r² = 0.9912)

The obtained results made it possible to calculate the amount of released ketoprofen for each series of mini tablets. Based on the obtained results,

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Mini-tablets IR</th>
<th>Mini-tablets SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketoprofen</td>
<td>12.5 mg</td>
<td>29.2 mg</td>
</tr>
<tr>
<td>Microcrystalline cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silica colloidal</td>
<td>37.5 mg</td>
<td></td>
</tr>
<tr>
<td>Sodium starch glycolate (Prosolv Easy Tab, JRS Pharma)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium stearyl fumarate</td>
<td>0.3 mg</td>
<td></td>
</tr>
<tr>
<td>Hypromellose K4M (Colorcon)</td>
<td></td>
<td>18.0 mg</td>
</tr>
<tr>
<td>Total</td>
<td>50 mg</td>
<td>60 mg</td>
</tr>
<tr>
<td>Hardness [N]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBH-420TD Erweka</td>
<td>103.1 ± 1.17</td>
<td>133.5 ± 3.24</td>
</tr>
<tr>
<td>Friability</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA3 Erweka</td>
<td>0.04%</td>
<td>0.17%</td>
</tr>
<tr>
<td>Disintegration time [s]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZT71 Erweka</td>
<td>60 ± 2</td>
<td></td>
</tr>
</tbody>
</table>
Multi-compartment systems were constructed by placing mini tablets in a capsule of hypromellose. The amount of released ketoprofen from the mini tablets and multi-compartment system were tested in vitro in the dissolution apparatus with a paddle (DT 70, Erweka), using the acceptor fluid replacement method (23). To avoid floating of multi-compartment systems on the surface of acceptor fluids aggravating elements (Sinkers) were used. The rotation speed of the agitator was 50/min while the water temperature in the bath was 37 ± 1°C. The volume of acceptor fluids was 900 mL. The following two acceptor fluids had been used in the study of multi-compartment systems:

- 0.1 M HCl - imitating the environment prevailing in the stomach,
- phosphate buffer, pH 6.8 – simulating the conditions in the small intestine.

Three types of multi-compartment systems were prepared, containing, respectively:

- IR + IRc – 2 immediate release mini tablets and 2 immediate release coated mini tablets. This model simulates the pulsatile release model in which half of the dose is released in the stomach and the other half in the small intestine;
- IR + 3SR – 1 immediate release mini tablet and 3 sustained release mini-tablets. This corresponds to a classic model of a drug of sustained action, wherein part of the substance, in initial dose form, is released in the stomach, and the other part as a maintenance dose is slowly released and absorbed in the small intestine;
- IRc + 3SR – 1 immediate release coated mini tablet and 3 sustained release mini-tablets where only a small, insignificant portion of ketoprofen is released from matrix tablets in the stomach while both, the initial dose and maintenance dose are released in the small intestine. This model can be proposed for the treatment of patients with damaged gastric mucosa and enables to avoid its irritation.

Studies on ketoprofen release from multi-compartment systems had been performed up to the 120th minute with the use of 0.1 M HCl as acceptor fluid and afterwards the releasing medium was changed to phosphate buffer pH 6.8.

RESULTS AND DISCUSSION

Use of Prosolv Easy Tab and Prosolv 90HD made it possible to carry out direct compression - the fastest and cheapest method of tabletting. Obtained mini tablets with ketoprofen were characterized by high hardness (103 ± 2.26 N for IR mini tablets and 133.5 ± 3.24 N for SR mini tablets) and low friability (0.04% and 0.17%, respectively). It should be noted, that a considerable hardness of IR mini tablets did not lead to unfavorable prolong of their disintegration time (60 ± 2 s) (Table 1).

Results of dissolution testing show clearly, that the pH is the critical factor determining the quantity of ketoprofen, which may be dissolved in the medium, after release from the mini tablets. Solubility of ketoprofen increases substantially with increasing
pH (16, 24). In 0.1 M HCl, after 120 min, was dissolved 20% of ketoprofen, released from IR mini tablets. In the phosphate buffer pH 4.5 was dissolved almost 70% and in phosphate buffer pH 6.8 about 90%. The results of ketoprofen release from mini tablets are given in Figures 1 and 2.

The effect of pH on the dissolution rate of ketoprofen is also significant. In the first 30 min of the study, was dissolved in a medium, respectively:

- In 0.1 M HCl - 6.09% of ketoprofen, representing about 30% of the total amount of ketoprofen, dissolved within 120 min.
- In buffer pH 4.5 - 34.26%, representing approximately 50% of the total amount of ketoprofen, dissolved within 120 min.
- In buffer pH 6.8 - 81.95%, which is almost 90% of the dose of ketoprofen, which completely dissolved during the test.

![Figure 2. Amount of released ketoprofen (% ± SD) from SR mini tablets in buffer pH 6.8 over 12 h (n = 12)](image)

![Figure 3. Amounts of released ketoprofen (% ± SD) from multi-compartment systems (n = 12)](image)
This clearly shows that a relatively small change in pH in the stomach may have a significant impact on the solubility and absorption of ketoprofen, and through it, on the time, after which a therapeutic effect is observed (16). The situation when the pH of the stomach is higher than the physiological, can take place, inter alia, during long term use of proton pump inhibitors (omeprazole, pantoprazole), as well as during the concomitant use of ketoprofen and antacids (magnesium hydroxide, aluminum hydroxide) (25).

In studies of ketoprofen release from sustained release mini tablets (SR), the effect of pH on the dissolution rate of released ketoprofen is also observed. However, the main factor, controlling the amount of ketoprofen released into the medium is the diffusion rate through the swollen matrix. The amount of dissolved ketoprofen increases with an increase of pH, but in any medium, an increase of its concentration is quite uniform. There is no rapid dissolving of substantial amount of the active substance in the first 30 min of the release, as it was observed in the IR mini tablets studies. In the initial stage, when the mini tablet absorbs the medium and forms a matrix, we can see the graph curve slightly flattened (Fig. 2). Only when the diffusion rate of ketoprofen to the medium stabilizes, the curve is steeper and close to a straight line.

In the study on the multi-compartment system #3 (Fig. 3), where the SR mini tablets are one of the components of the system, the effect of pH change on the release rate of ketoprofen is also clearly visible. For 120 min, about 7% of ketoprofen, originating from IR and SR mini tablets was dissolved, which is an initial dose. When in the 120th minute the medium was changed (the vertical, dashed line) to phosphate buffer pH 6.8, the rest of the initial dosage rapidly dissolved. The remainder of ketoprofen contained in SR mini-tablets was released more slowly than would be apparent from the results, obtained in the study on SR mini tablets in a phosphate buffer pH 6.8 (Fig. 2). From 150th to 240th minute of testing, the curve has a flatter course than in the distal section. This means, that ketoprofen releases from the matrix slower, despite the relatively long residence of SR mini-tablets in the buffer, with the best observed solubility of the active substance. It seems that this is due to soaking mini-tablets by hydrochloric acid during their residence in 0.1 M HCl solution. Formed matrix, restricts the exchange of ketoprofen with the surrounding buffer. It was not until 240th minute that the curve is of steeper course, which may indicate a change in the permeability of the matrix. This leads to the suspicion that in a clinical conditions similar phenomenon may occur. When a tablet administered to a patient is surrounded by chyme with a low pH, the diffusion rate of active substance from the dosage may be lower than in vitro. Summary of the results of the active ingredient release from multi-compartment systems, as well as types of analyzed multi-compartment systems are given in Figure 3.

Comparison of the amounts of released ketoprofen from the IR and the IRc mini tablets, shows
significant difference in the release rate of ketoprofen in phosphate buffer pH 6.8 (Fig. 4). The percentage of the released ketoprofen at 30th and 60th min is higher for IR mini tablets than for IRc mini tablets, although the compositions of both are the same, and the envelope covering the core of the IRc mini tablet dissolved very quickly. Amounts of ketoprofen released in the 90th and 120th min of a study are similar, and the differences between them are statistically insignificant. The observed phenomenon indicates that the presence of the enteric coating on the core has an impact on the release rate of ketoprofen. All data are shown as the mean values ± standard deviation and were evaluated with Statistica 10.0 (StatSoft, Poland).

The proposed design of multi-compartment systems, based on the results of the mini tablets ketoprofen dissolution testing, makes it easier to design the composition of the dosage form. Thanks to this it is possible to adjust the release profile of the active substance to the individual patient, which meets the expectations of personalized medicine (26). Preparation of the proposed form of drug in the present embodiments, can be also less expensive than developing a complex form of the drug in tablet form (27, 28). In addition, dividing the total dose into smaller subunits accelerates the release of the active substance compared to the release of the same dose, contained in one tablet (29). This is particularly important in the treatment with NSAID and helps to avoid local irritation of the gastric mucosa by slowly disintegrating tablet.

CONCLUSIONS

The factor limiting the amount of available ketoprofen at the absorption place is pH of the environment. A relatively small change in pH in the stomach may have a significant impact on the solubility and absorption of ketoprofen, and through it, on the time, after which a therapeutic effect is observed. Multi-compartment systems are superior to traditional drug forms in terms of more efficient control of release and absorption place. Preparation of the proposed form of drug in the present embodiments, can be also easier and less expensive than developing a complex form of the drug in tablet form.

REFERENCES


Received: 19.03.2015
Indomethacin, a commonly used non-steroidal anti-inflammatory drug (NSAID), shows slow dissolution and high permeability through stomach. It is used to reduce pain/swelling involved in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, tendinitis, gout, bursitis and headaches. According to the Biopharmaceutics Classification System, indomethacin is classified as class II drug in which the dissolution is rate limiting step in the process of drug absorption. Water-insoluble drugs often show weak bioavailability, improvement in solubility and/or dissolution rate is a very important consideration for drug development process (1-3). Micronization, solid dispersion, solubilization by surfactants and use of complexing agents are some of the methods which have been used to enhance dissolution of water insoluble drugs. Previous studies have found that interactive mixtures prepared from finally divided poorly water soluble drug particles with coarse carrier increased the dissolution rate, compared with the micronized particles alone, but agglomeration was not totally eliminated (4-7). Research in this area suggested that the use of micronized excipient as a ternary additive might enhance the dissolution of agglomerated micronized drugs (7-10). There is evidence from the literature that addition of fine lactose to the interactive mixture containing indomethacin and PVP, while the cloud point of 1% PVP containing ammonium sulfate was not affected by the addition of lactose.

**DISSOLUTION AND COMPATIBILITY STUDY OF BINARY AND TERNARY INTERACTIVE MIXTURES OF INDOMETHACIN: COMPARISON WITH COMMERCIAL AVAILABLE CAPSULES**

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**Abstract:** The main objective of this work was to use Weibull distribution function and Baker-Lonsdale models to study the dissolution kinetics of prepared binary and ternary interactive mixtures containing indomethacin in comparison with three commercially available capsules of indomethacin, namely, Rothacin®, Indomin® and Indylom®. Differential scanning calorimetry (DSC) in conjunction with cloud point method was used to study the compatibility of indomethacin with polyvinylpyrrolidone (PVP) and lactose and to provide an explanation(s) for the insignificant increase in dissolution rate observed in the ternary interactive mixture as well as for the reduction in the dissolution rate observed from the binary system in our previous study. Results showed that the Weibull distribution function equation was the best fit to the dissolution data for all formulations used in this study. DSC curves showed that the decrease in dissolution rate from the binary and ternary interactive mixtures was due to incompatibility of indomethacin with PVP. Also DSC curves showed that lactose was compatible with indomethacin and that lactose was used as excipient in two commercial products (Rothacin® and Indylom®). Results from the cloud point method showed that the addition of indomethacin to 1% PVP solution containing ammonium sulfate (with cloud point at 76°C) reduces the cloud point of PVP indicating that there is an interaction between indomethacin and PVP, while the cloud point of 1% PVP containing ammonium sulfate was not affected by the addition of lactose.

**Keywords:** indomethacin, ternary interactive mixture, compatibility, polyvinylpyrrolidone, lactose, DSC

Indomethacin, a commonly used non-steroidal anti-inflammatory drug (NSAID), shows slow dissolution and high permeability through stomach. It is used to reduce pain/swelling involved in rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, tendinitis, gout, bursitis and headaches. According to the Biopharmaceutics Classification System, indomethacin is classified as class II drug in which the dissolution is rate limiting step in the process of drug absorption. Because water-insoluble drugs often show weak bioavailability, improvement in solubility and/or dissolution rate is a very important consideration for drug development process (1-3).

Micronization, solid dispersion, solubilization by surfactants and use of complexing agents are some of the methods which have been used to enhance dissolution of water insoluble drugs. Previous studies have found that interactive mixtures prepared from finally divided poorly water soluble drug particles with coarse carrier increased the dissolution rate, compared with the micronized particles alone, but agglomeration was not totally eliminated (4-7). Research in this area suggested that the use of micronized excipient as a ternary additive might enhance the dissolution of agglomerated micronized drugs (7-10). There is evidence from the literature that addition of fine lactose to the interactive mixture containing indomethacin resulted in an increase in the *in vitro* dissolution of drug from the binary mixture (11, 12). However, in our previous study, we showed that the release of indomethacin was reduced from binary system containing 10% PVP as additive and the release was increased after the addition of 10% fine lactose to the binary system to prepare the ternary interactive mixture. In the same study, results showed that the release of indomethacin from commercially available capsules was higher (12). It is well known that the decrease in the dissolution rate occurs due to many factors such as particle size of drug, type and amount of excipients used, particle strength, drug morphology, drug - excipient incompatibility, drug-

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fine lactose ratio and many other factors (12-19). A number of experimental techniques (i.e., DSC, X-ray powder diffraction, optical and electron microscopy, FT-IR spectroscopy, cloud point method, equilibrium dialysis and solubility measurement etc.) have been used to investigate the interaction between drug and excipients (20-24). DSC is a quick technique to investigate excipient - drug incompatibility derived from the appearance, disappearance or shifts of peaks and/or variation in the corresponding ∆H (enthalpy of transition). The cloud point method is a simple and good reproducible method used to study the interaction between low molecular weight compounds and other water soluble macromolecules (25, 26).

The aim of this work was to study the dissolution kinetics of all formulations (binary system, ternary interactive mixture and three commercially available capsules) by using Weibull distribution function and Baker-Lonsdale models. DSC was used to study the possibility of drug – excipient interaction and to give an explanation(s) for the release reduction observed in the binary and ternary interactive mixtures containing indomethacin. In addition, DSC curves obtained for the

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Weibull equation</th>
<th>Baker-Lonsdale model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²</td>
<td>Time scale (a)</td>
</tr>
<tr>
<td>Indomin</td>
<td>0.97</td>
<td>1.44</td>
</tr>
<tr>
<td>Indylon</td>
<td>0.95</td>
<td>1.97</td>
</tr>
<tr>
<td>Rothacin</td>
<td>0.95</td>
<td>2.10</td>
</tr>
<tr>
<td>Binary system</td>
<td>0.85</td>
<td>2.52</td>
</tr>
<tr>
<td>Ternary mixture</td>
<td>0.98</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Figure 1. A linear plot of dissolution data in accordance with the Weibull distribution function model: Indomin (○), Indylon (□), ternary interactive mixture (×), binary mixture (△) and Rothacin (■)
Table 2: Data for melting onset, melting point, endset of melting and enthalpy of fusion obtained from DSC curves for micronized indomethacin mixtures and commercially available capsules.

<table>
<thead>
<tr>
<th></th>
<th>Indomethacin melting onset $T_{m/m}$ °C</th>
<th>Indomethacin peak of melting $H_{m/°C}$</th>
<th>Indomethacin endset of melting $T_{m/°C}$</th>
<th>Indomethacin enthalpy of fusion J/g</th>
<th>Lactose onset $T_{m/°C}$</th>
<th>Lactose peak $H_{m/°C}$</th>
<th>Lactose endset $T_{m/°C}$</th>
<th>Lactose enthalpy J/g</th>
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</thead>
<tbody>
<tr>
<td>Indomethacin (micronized)</td>
<td>159.64</td>
<td>162.27</td>
<td>166.16</td>
<td>93.86</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin + PVP (90 : 10)</td>
<td>156.8</td>
<td>160.13</td>
<td>163.37</td>
<td>101.09</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indomethacin + 50% PVP (50 : 50)</td>
<td>133.82</td>
<td>144.88</td>
<td>160.97</td>
<td>49.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>Indomethacin + Lactose (90 : 10)</td>
<td>159.27</td>
<td>161.78</td>
<td>164.75</td>
<td>65.06</td>
<td>*Deh.145.51</td>
<td>148.16</td>
<td>151.31</td>
<td>2.6</td>
</tr>
<tr>
<td>Indomethacin + Lactose (50 : 50)</td>
<td>159.01</td>
<td>161.67</td>
<td>166.0</td>
<td>61.52</td>
<td>*Deh. 144.63</td>
<td>148.26</td>
<td>152.74</td>
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<tr>
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<td>-</td>
<td>-</td>
<td>*Deh. 140.16</td>
<td>145.92</td>
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<td>176.62</td>
<td>3.38</td>
<td>*Deh. 155.23</td>
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<td>160.30</td>
<td>2.75</td>
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<tr>
<td>Rothacin</td>
<td>-</td>
<td>157.0</td>
<td>-</td>
<td>-</td>
<td>*Deh. 144.42</td>
<td>147.99</td>
<td>152.59</td>
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<tr>
<td>Indomin</td>
<td>148.73</td>
<td>154.89</td>
<td>157.67</td>
<td>6.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Indylon</td>
<td>157.95</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*Deh. 144.25</td>
<td>149.08</td>
<td>155.06</td>
<td>70.09</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*Deh. 143.17</td>
<td>148.74</td>
<td>154.23</td>
<td>31.46</td>
</tr>
</tbody>
</table>

*Dehydration, **Decomposition
commercially available capsules of indomethacin (Rothacin®, Indomin® and Indylon®) were compared with the binary and ternary interactive mixtures prepared in this study. Finally, the cloud point method was used to support results obtained from DSC concerning drug – excipient interaction.

EXPERIMENTAL

Materials

Commercial indomethacin capsules (Rothacin®, Indomin® and Indylon®) were purchased from the Saudi market. Lactose monohydrate and povidone (PVP) were obtained from Sigma, USA. Indomethacin was donated by Deef (Deef, Qassim, Kingdom of Saudi Arabia) and originally manufactured by Albemarle (USA). Fine lactose was prepared by manually grinding lactose monohydrate using a mortar and pestle and then sieving it using a standard stainless steel sieve (45 µm).

Preparation of binary and ternary interactive mixtures

Lactose-povidone granules and ternary interactive mixture were prepared as described by Allahham and Maswadeh (12). In brief, the lactose-povidone granules were prepared from lactose and povidone in a 9 : 1 ratio by wet granulation using a 10% (w/w) povidone solution. The wet granules were tray-dried and dry granules were lightly comminuted using a mortar and pestle and then sieved to obtain the required size fraction (125–250 µm). The binary interactive mixtures were prepared by weighing the required amount of micronized indomethacin (with volume mean diameter of 12.20 µm) and placing it between two equal layers of the coarse carrier in a glass vial, making the total weight up to 2.5 g with carrier and then shaken vigorously for 5 min by hand. Ternary interactive mixtures were prepared by weighing the required amounts of drug and the ternary additive (fine lactose with volume mean diameter of 22.93 µm) and placing them between two equal layers of coarse carrier in a glass vial, making the total weight up to 2.5 g with carrier. The glass vial was then turned over and shaken in the same way as described for the binary interactive mixture.

In vitro dissolution study

In vitro dissolution for all indomethacin capsules (formulated and commercial) was evaluated as described in Allahham and Maswadeh (12).

Differential scanning calorimetry (DSC)

DSC runs of all materials and mixtures used for the preparation of binary and ternary interactive mixture as well as commercially available capsules were performed by using DSC-60 (Shimadzu, Japan). The thermal profiles of these materials were measured by DSC using 4 mg of sample in crimped aluminum pans and a heating rate of 10°C/min.

Cloud point study

The cloud point method was used to study the interaction between indomethacin and PVP.
Reduction of cloud point for 1% PVP solution was performed by the addition of ammonium sulfate. The cloud point temperatures (CPT) were obtained by placing the test tubes each containing 10 mL of 1% PVP solution containing ammonium sulfate with 2, 4, 6 and 8 mg indomethacin into a temperature-controlled bath. The sample solutions were heated to a temperature where cloudy appearance was visualized. Typically, it was observed that the solution turns completely turbid within one degree. The temperature at the first sign of the turbidness was taken as the CPT.

RESULTS AND DISCUSSION

The Weibull distribution function and Baker-Lonsdale equations were used to study the mechanism of drug release from the binary system, ternary interactive mixture and three commercially available capsules of indomethacin namely, Rothacin®, Indylon® and Indomin®.

The Weibull distribution function is a general empirical equation that can be successfully applied to almost all kinds of dissolution curves and is commonly used in these studies (27-31). The linear form of Weibull equation is expressed as (32):

\[
\log [-\ln (1 - m)] = b \log (t - T) - \log a
\]  

In this equation, the scale parameter, \(a\), defines the time scale of the process. The shape parameter, \(b\), characterizes the curve as either exponential (\(b = 1\)) (case 1), sigmoid (\(b > 1\)) (case 2), or parabolic, with a higher initial slope and after that consistent with the exponential (\(b < 1\)) (case 3).

The shape parameter (\(b\)) is obtained from the slope of the line and the scale parameter - \(a\), is estimated from the intercept. The parameter, \(a\), can be replaced by the dissolution time, \(T_d\), that is defined by \(T_d = (a)^{1/b}\) and is equivalent to \(m = 0.632\), \(T_d\) represents the time interval necessary to dissolve or release 63.2% of the drug present in the pharmaceutical dosage form (32).

The dissolution data of all formulations were plotted in accordance with the linear form of Weibull distribution function (Fig. 1). Results show that Weibull distribution function equation fit to the dissolution data for all formulations with a linear regression coefficient of determination \(r^2\) values between 0.85 and 0.98 (Table 1). The curve of dissolution for all formulations was parabolic, with a
higher initial slope and after that consistent with the exponential \((b < 1)\) as shown in Table 1. The dissolution time \((T_d)\) was 3.3, 5.0, 5.8, 7.3 and 12.4 min for Indomin®, Indylon®, Rothacin®, ternary and binary interactive mixtures, respectively (Table 1). Dissolution time \((T_d)\) indicates that the release rate of indomethacin was faster from commercially available capsules in compression with the ternary and binary interactive mixtures.

Baker-Lonsdale model was developed by Baker and Lonsdale (1974) from the Higuchi model and describes the drug controlled release from a spherical matrix, microcapsules or microspheres, being represented by the following linear expression (32-35):

\[
\frac{3}{2} \left[ 1 - \left(1 - \frac{M_t}{M_\infty}\right)^{2/3} \right] - \frac{M_t}{M_\infty} = Kt \tag{2}
\]

where the release constant \((K)\) corresponds to the slope. \(M_t\) is the drug released amount at time \(t\) and \(M_\infty\) is the amount of drug released at an infinite time.

The dissolution data for all formulations were plotted in accordance with the Baker-Lonsdale equation (Fig. 2). Results show that Baker-Lonsdale equation fits to the dissolution data with a linear regression coefficient of determination \(r^2\) values between 0.67 to 0.96 indicating that Weibull distribution function model is best described by the dissolution data for all formulations with higher \(r^2\) values (Table 1).

To study the possibility of drug - excipient interaction, DSC curves for all formulations and a mixtures of micronized indomethacin - PVP/or lactose (90 : 10% w/w and 50 : 50 w/w) were obtained by using DSC-60 (Shimadzu, Japan).

Table 2 and Figure 3 show that pure micronized indomethacin has a melting peak at 162.27°C with enthalpy of fusion equal to 93.86 J/g. Results indicate that PVP was interacted with micronized indomethacin in all concentrations used (Table 2 and Fig. 3). More specifically, the addition of 10% PVP produces a decrease in melting peak of micronized indomethacin by 2.14°C and at higher concentration of PVP (50%) the enthalpy of fusion was dramatically decreased and a decrease in the

Figure 5. DCS curves for (a) indomethacin, (b) binary mixture and (c) ternary interactive mixture
melting peak of micronized indomethacin by 17.39°C was observed. This finding appears to be in agreement with previous studies, where it was found that PVP was incompatible with a wide range of active pharmaceutical ingredients such as oxprenolol, atenolol, ibuprofen, indomethacin, ranitidine and raloxifene hydrochloride (36-38). It has been reported that PVP mainly interacts with drug molecules by electrostatic bonds (ion to ion, ion to dipole, dipole to dipole) along with Van der Waals forces and H-bonds. Drug-excipient interaction through H-bond interaction of indomethacin-PVP may produce drug - excipient interaction (39-43).

Mixtures of micronized indomethacin-micronized lactose (90 : 10% w/w and 50 : 50 w/w) were used to study the indomethacin - lactose interaction. Table 1 and Figure 4 show that the addition of 10% fine lactose to micronized indomethacin decrease the melting peak of indomethacin by 0.49°C and the addition of 50% fine lactose to the micronized indomethacin produces a decrease in the melting peak of indomethacin by 0.6°C. Micronized indomethacin-fine lactose interaction was not found and the decrease in the melting point was insignificant.

In case of binary system (Fig. 5b) composed from 20% micronized indomethacin and 80% fine lactose-PVP (9 : 1) used in this study, a decrease of 7.1°C of the melting peak of micronized indomethacin was observed and the peak was very close to the melting peak of lactose indicating a very strong interaction of indomethacin with the mixture lactose – PVP (9 : 1 w/w).
The effect of the addition of fine lactose 20% to the binary system was also investigated. Table 1 and Figure 5c show that the addition of 20% fine lactose to the binary system has a different type of indomethacin-excipient interaction by increasing the melting peak of indomethacin from 162.2 to 171.8°C.

Commercially available capsules of indomethacin (Rothacin®, Indomin® and Indylon®) were used to compare the melting point for micronized with non-micronized indomethacin and to study the type of excipient(s) used in the three commercially available formulations. Table 1 shows that the melting peak of Rothacin® and Indylon® was similar with insignificant differences. This similarity was supported by the DSC curves obtained for Rothacin® and Indylon showing that lactose was used as excipient in both formulations (Fig. 6a and 6c). In case of Indomin® a small peak at 109.72°C and a broad peak between 30-70°C were observed and represent some additives (excipient) in the formulation, but lactose was not found. However, it is important to note that the initial release rate from Indomin® was faster than the release rate from Rothacin® and Indylon as well as from the binary and ternary interactive mixtures prepared in this study.

Figure 7. Reduction of cloud point of 1% PVP by the addition of ammonium sulfate

\[
y = -11.579x + 202.88 \\
R^2 = 0.9881
\]

Figure 8. The effect of indomethacin in the cloud point of 1% PVP containing ammonium sulfate
solution rate observed from Indomin®, Rothacin® and Indylon® in comparison with the binary and ternary interactive mixtures may be due to some additives in their formulations, such as non-ionic surfactant that enhance dissolution. This additive was observed in the DSC curves, by a small peak at 110°C for Indomin®, Rothacin® and Indylon® (Fig. 6).

The cloud point method is a simple and good reproducible method used to study the interaction between low molecular weight compounds and other macromolecules (44, 45). It was reported that the cloud point of PVP is > 100°C and can appeared by addition of the least amount of ammonium sulfate and that the cloud point of PVP aqueous solutions lowered linearly with increasing concentration of ammonium sulfate. The amount of ammonium sulfate needed to coacervate the solution of 1% PVP is shown in Figure 7. Results show that the addition of indomethacin to 1% PVP solution containing ammonium sulfate (with cloud point at 76°C) reduces the cloud point of PVP indicating that there is an interaction between indomethacin and PVP (Figs. 8, 9). A second cloud point was observed after cooling due to salting out of indomethacin and appeared at 35°C and 40°C after the addition of 4 mg and 6 mg of indomethacin, respectively (Figs. 8, 9). The cloud point of 1% PVP containing ammonium sulfate was not affected by the addition of lactose.

CONCLUSIONS

It can be concluded that the reduction in the dissolution rate from the binary and ternary interactive mixtures of indomethacin in our previous study was due to the drug – PVP interaction. This interaction was also confirmed by the cloud point method that showed that the cloud point of 1% PVP containing ammonium sulfate was reduced after indomethacin addition. Therefore, PVP must be avoided in the preparation of binary and ternary interactive mixtures of indomethacin to prevent drug - excipient interaction. Also it can be concluded that indomethacin was compatible with lactose and that lactose was used as excipient in Rothacin® and Indylon®, while PVP was not detected.

REFERENCES


Figure 9. Photographs of (a) 1% PVP containing ammonium sulfate, (b) complete cloud sample represent the cloud point of 1% PVP containing ammonium sulfate, (c) starting cooling the sample, (d) separation of sample in two layers, (e) cloud sample after cooling due to salting out of indomethacin

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Although largely preventable, dental caries and periodontal disease are the two biggest threats to oral health, and are among the most common chronic diseases in children and adolescents. Dental caries is the most common chronic disease in children; it is about five times as common as asthma and seven times as common as hay fever in adolescents. Dental caries also affects adults, 90% of people over the age of 20 have some degree of tooth-root decay. Despite the decline in the prevalence of dental caries in children in the western countries, caries in pre-school children remain a problem in both developed and developing countries (1, 2).

Moreover, the most common cause of tooth loss among adults is untreated periodontal disease. Fifty-three million people live with untreated tooth decay in their permanent teeth. Strikingly, one-quarter of adults aged 65 years and older have lost all of their teeth due to untreated oral disease tooth loss (3). Having in regard duration of treatment, restorative dental material could be divided into two groups: temporary and long-term used. A great number of temporary restorative materials have been used to seal access cavities, including gutta percha, zinc oxide-eugenol (ZOE) cements and their coproducts, zinc phosphate cements, glass ionomer materials and composite resins (4). An example of such fillings at the dental market are ZOE mixed with thymol (ThymodentinÆ) and Coltosol FÆ (5-7).

DEVELOPMENT AND VERIFICATION OF NEW SOLID DENTAL FILLING TEMPORARY MATERIALS CONTAINING ZINC. FORMULA DEVELOPMENT STAGE

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Abstract: Caries is the most popular problem affecting teeth and this is the reason why so many temporary dental filling materials are being developed. An example of such filling is zinc oxide paste mixed with eugenol, Thymodentin and Coltosol FÆ. Zinc-oxide eugenol is used in dentistry because of its multiplied values: it improves healing of the pulp by dentine bridge formation; has antiseptic properties; is hygroscopic. Because of these advantages compounds of zinc oxide are used as temporary fillings, especially in deep caries lesions when treatment is oriented on support of vital pulp. Temporary dental fillings based on zinc oxide are prepared ex tempore by simple mixing powder (Thymodentin) and eugenol liquid together or a ready to use paste Coltosol FÆ. Quantitative composition depends mainly on experience of person who is preparing it, therefore, exact qualitative composition of dental fillings is not replicable. The main goal of the study was to develop appropriate dental fillings in solid form containing set amount of zinc oxide. Within the study, the influence of preparation method on solid dental fillings properties like mechanical properties and zinc ions release were examined.

Keywords: dental fillings, tableting, anodic stripping voltammetry, zinc

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Effects on microtensile bond strength of adhesives to dentin after 24 h of exposure (12). Temporary dental fillings based on zinc oxide are prepared ex tempore simply by mixing powder (Thymodentin®) and liquid eugenol together. Coltosol® is a ready-to-use paste which hardens in the environment of oral cavity with good coronal seal ability (13). In the first case where Thymodentin is being used, quantitative composition depends mainly on experience of person who prepares it. Exact qualitative composition of dental fillings is not replicable (5-7). Despite its obvious advantages there are described cases of allergic reactions caused by dental fillings. They results from the continuous release of a different - depending on the type of filling - elements, as well as the individual sensitivity. There are also described, others like: mucosal lichen planus (14) cases occurring in the oral cavity, palmoplantar pustulosis (15), follicular rash (maculopapular rash) covering a larger area of the body (16, 22), or a case of erythema, edema and lumpy rash all over the body occurring in individuals due to an allergic reaction to zinc released from the four applied dental fillings (8). The main goal of the study was to develop appropriate dental fillings in solid form containing set amount of zinc oxide. Within the study, the influence of preparation method on solid dental fillings properties were examined. In order to check the chemical stability of the dental fillings, analysis of release of Zn(II) from prepared dental fillings was done.

EXPERIMENTAL

Materials and equipments

The materials used were zinc oxide (POCh), eugenol (Merck), Neusilin US2 (Fuji Chemicals), talc, hydroxypropylmethylcellulose (Pharmacoat 603) (Shin Etsu), colloidal silicon dioxide (Cab-O-Sil), calcium phosphate (Pharma-Zentrale GmbH, Germany), Coltosol® F (Coltene, Switzerland), Thymodentin (Chema-Elektromet, Poland). Equipment used: laboratory kneader LK 5 Erweka, Kube mixer KB Erweka (Germany), dry K 100 (Premed, Polska), tabletting machine Korsch EK0, hardness tester (VenKel VK200), friabilator (Pharma Test PTF E, Germany), angle of repose apparatus, angle of slight apparatus, texture analyzer EZ-SX (Shimadzu, Japan), a multipurpose Electrochemical Analyzer M161 with the electrode stand M164 (both MTM-ANKO, Poland) equipped with a classical three electrode quartz cell, volume 5 mL, consisting of Controlled Growth Mercury Drop Electrode (CGMDE), as the working electrode, a double junction reference electrode Ag/AgCl/ 3M KCl with replaceable outer junction (2.5 M KNO₃) and a platinum wire as an auxiliary electrode. The apparatus was connected to a personal computer with EA9 software. Other materials used for zinc ions determination were: four times distilled water with a conductivity of less than 1 µS/cm, (distillation apparatus SZ-97A, Chemland, Poland); HNO₃ (Merck, Suprapur®); a standard stock solution of Zn (prepared by proper dilution of solution with a concentration 1 g/mL OUM-7 Łódź), supporting electrolyte (prepared by dissolving KNO₃ (Merck, Suprapur®) 3M); and a solution for imitating saliva pH 6.7 (0.400 g of potassium dihydrophosphate, di-sodium hydrophosphate 0.5200 g, 0.6600 g of potassium thiocyanate, 3.0000 g of sodium bicarbonate, 1.4000 g sodium chloride, 0.2600 g of urea, 2.4000 g of potassium chloride, dissolved in quadruple distilled water in a 1 L volumetric flask).

Preparation of temporary solid dental fillings

Development formula was done by dry granulation and wet granulation methods. Regardless on method of preparation each filling contained 90.0 ± 2% zinc oxide. Dry granulation consists of compaction step followed by a milling step (18, 19). Weighted powder preparation was as follows: zinc oxide, calcium phosphate, and colloidal silicon dioxide were mixed together in Kube mixer KB Erweka. Slugging was performed on a single punch tabletting machine Korsch EK0 using flat punches (diameter 20 mm). The slugs were manually crushed, talc was added and solid dental fillings were obtained in tabletting process.

To obtained solid dental fillings wet granulation was introduced as a second method of preparation (20, 21). The wet granulation formulation composed of zinc oxide, which was wetted using 15% Pharmacoat 603 as a binder solution, magnesium aluminoemetasilicate, calcium phosphate and talc. The granulate was obtained in laboratory kneader LK 5, dried using KCW 100 dryer, until up to 0.1% weight loss on drying was achieved. To the granulate, magnesium aluminoemetasilicate and calcium phosphate were added and mixed together. In the final step, talc was added and mixed. Solid dental filling were obtained in tabletting process.

The compressed dental fillings obtained by both dry and wet granulation were prepared during tabletting process using single punch tabletting machine Korsch EK0 equipped with flat punches (4 mm diameter), tablet weight was 60 mg. They were also characterized by required physical properties that allowed manual crushing just before mixing.
with set amount of eugenol and application into teeth.

For comparison purposes of zinc ions release, ready to use temporary dental fillings: Coltosol® F and Thymodentin were manually compressed into tablet shape dental filling. They characterized the same shape, diameter and weight as fillings prepared by dry and wet granulation process.

**Testing of final mixtures and solid dental fillings**

Properties of final mixtures and temporary solid dental fillings obtained in tableting process were verified by application of appropriate methods. Bulk and tapped density, as well as angle of repose were determined according to Polish Pharmacopoeia (FP X) procedures. Weighted amount of final mixtures was load into 100 mL volume cylinder. Bulk density was calculated as a mass of powder divided by the bulk volume, expressed in g/cm³. Tapped density of final mixtures was determined by filled 100 mL volume cylinder, mounted on a tapping platform and tapped until constant height was obtained. The parameter is expressed in g/cm³ as a mass of powder divided by volume after tapping. Based on bulk and tap density measurement results, Hausner ratio and compressibility index were calculated based on the following equations:

- Hausner ratio = tapped density / bulk density
- Compressibility index = 100 × (tapped density – bulk density) / tapped density

Angle of repose is a three dimensional cone like angle formed by the material during determination. It was calculated after the final mixture was passed through sieve on the polished surface 60 mm diameter. Angle of slight is defined as the angle to which a polished metal plate must be tilted of powder to start sliding down the plate.

The physical characteristics, hardness and friability of obtained solid dental fillings were also verified according to Polish Pharmacopoeia (FP X) procedures. Hardness was determined by measuring of 10 dental fillings using hardness tester apparatus VenKev VK200. Friability was determined by introduction of 6.5 g of dental fitting into friabilator Pharma Test PTF E at 25 rpm for 4 min. After the test, weight loss was calculated. The force needed to crushed solid dental fillings was determined using texture analyzer EZ-SX, after braking a dental filling by single flat probe 10 mm diameter at a speed of 10 mm/min.

**Determination of zinc ions released**

The study determined the concentration of zinc ions released from the selected different types of dental fillings, using the DP ASV (differential pulse anodic stripping voltammetry) method (22, 23). Tablets prepared as dental fillings were placed in an artificial saliva solution for 2, 48 and 168 h. After this time, the solutions were decanted from above the tablets, and the concentration of zinc ions was determined using the method of anodic stripping voltammetry. The proposed voltammetric method using CGMDE allowed the determination of zinc (20 s deposition time) with LOD = 0.64 µg/L and LOQ = 1.92 µg/L. The slope of regression curve was 2.429 ± 0.036 nA/µg/L, with correlation coefficients r = 0.9997. The precision of the method described by the RSD (relative standard deviation) of a sample was not worse than 16.58% (n = 9). The recovery of selected dental filling samples ranged from 94.7 to

<table>
<thead>
<tr>
<th>Type of dental filling</th>
<th>2 h [µg/mL]</th>
<th>48 h [µg/mL]</th>
<th>168 h [µg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>DGF (dry granulation fillings)</td>
<td>1.17</td>
<td>1.40</td>
<td>2.15</td>
</tr>
<tr>
<td>WGF (wet granulation fillings)</td>
<td>0.40</td>
<td>0.99</td>
<td>1.51</td>
</tr>
<tr>
<td>Thymodentin</td>
<td>36.15</td>
<td>55.61</td>
<td>73.04</td>
</tr>
<tr>
<td>Thymodentin + water</td>
<td>0.42</td>
<td>0.40</td>
<td>2.03</td>
</tr>
<tr>
<td>Zinc oxide</td>
<td>3.90</td>
<td>4.69</td>
<td>20.78</td>
</tr>
<tr>
<td>Zinc oxide-eugenol</td>
<td>1.13</td>
<td>1.28</td>
<td>1.30</td>
</tr>
<tr>
<td>Coltosol F®</td>
<td>7.28</td>
<td>10.36</td>
<td>54.52</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

For the development of solid dental fillings the following requirements should be considered: content uniformity of zinc ions loading in the unit, appropriate mechanical properties of tablet shaped dental fillings that provide the possibility of manual pulverization and mixing with eugenol immediately before application into teeth decay.

Preliminary studies showed that direct compression method is not appropriate in temporary solid dental filling formula development process, so the dry granulation and wet granulation manufacturing methods were chosen and developed.

For both dry granulation as well as wet granulation manufacturing process, 20 different formula compositions were verified. The best formula gained for each method composition was chosen. The final mixture obtained by wet granulation process characterized higher bulk density and better flowability properties than final mixture obtained by dry granulation method (Table 2).

Better properties, regardless qualitative composition, are caused by influence of technological process. During wet granulation increase of density is more effective than in dry granulation. Binder solution caused that particles are connected and sticking together while in granulation process, during drying stage the water evaporates. The values of Hausner ratio and compressibility index show that flowability of final mixtures are also more favorable after wet granulation process. The influence of the type of process on flowability properties is also supported by angle of repose and angle of slight values. This parameters are better for final mixture gained by wet granulation process. Improvement of mixture properties influence on better characteristic of obtained temporary solid dental fillings.

The dental fillings obtained by dry granulation (DGF) and wet granulation (WGF) showed the differences in mechanical properties. WGF are harder and less fragile, their hardness is 32 N, in comparison to DGF characterized by hardness value 27 N. The hardness values were obtained from hardness tester. Friability of solid dental filling obtained by wet granulation method was 2.3%, dental filling manufactured by dry granulation process was completely crushed after friability study. Analysis proceeded using texture analyzer confirmed higher mechanical resistance of WGF in comparison to DGF solid dental filling. To crush this first, 3-fold higher force, with value of 150 N, should be applied. Given that developed compressed temporary solid dental fillings are going to be manually crushed and mixed with eugenol just before application into teeth, they should be soft enough. The mechanical properties of obtained solid dental fillings by both dry and wet granulation method allow to apply this procedure. They are soft enough to be manually pulverized. However, on the other hand, tableting at higher compression force to obtain harder dental fillings may be difficult because of capping occurred during the process.

Except of physical properties differences, solid dental fillings were also characterized by different amount of zinc ions dissolved into the artificial saliva solution which was also depending on the manufacturing method. The highest concentration was determined in the case of tablets made by dry granulation, incubated in artificial saliva for 168 h - it was 2.15 µg/mL dissolved. The quantity of zinc in the case of identical incubation time for the fillings prepared by wet granulation was nearly 1.5 times lower. The lowest concentration was observed in the 2-h incubation tablets prepared by wet granulation.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Granulation method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dry</td>
</tr>
<tr>
<td>Bulk density [g/cm³]</td>
<td>1.55</td>
</tr>
<tr>
<td>Tapped density [g/cm³]</td>
<td>1.98</td>
</tr>
<tr>
<td>Angle of repose [°]</td>
<td>40</td>
</tr>
<tr>
<td>Angle of slight [°]</td>
<td>29</td>
</tr>
<tr>
<td>Hausner ratio</td>
<td>1.28</td>
</tr>
<tr>
<td>Compressibility index</td>
<td>21.74</td>
</tr>
</tbody>
</table>
and amounted to 0.40 µg/mL. In both of parts DGF and WGF was observed that zinc concentration increased with length of incubation time.

The compiled data show that the concentration of zinc in the solution above the tablets increases over time. There was a significantly smaller amount of zinc released from the formulation with water and Thymodentin compared to compressed powder Thymodentin. The reason for this may be the curing of the formulation under the influence of water. The release of zinc from the dental filling is the result of compression the zinc oxide and eugenol and is maintained at a practically constant value from the start of the test up to 168 h. For filling containing compressed zinc oxide, after 168 h, the amount of zinc released is over 5 times higher compared to the sample subjected to 2 h of incubation in a solution of artificial saliva. Significant differences in the amounts of released zinc can be explained by the formation of complexes zinc oxide - eugenol.

The biggest difference between the amount of zinc released in the initial test period - after 2 h, and the end point of time - 168 h was observed for self-hardening paste Coltosol F. The amount of released zinc from Coltosol F increased 7.5 times. After 2 h of placement of a tablet from dental filling in artificial saliva solution, the majority of zinc was released from the tablet made from formulation Thymodentin, for which the concentration became 36.15 µg/mL. The high value of the concentration of zinc was also obtained in the case of self-hardening paste Coltosol F (7.28 µg/mL), but this value was almost five times lower than that for formulation Thymodentin. Low concentrations after 2 h was obtained for tablets made from a mixture formulation Thymodentin and water (0.42 µg/mL).

Similar conclusions can be drawn based on the analysis of the decanted solution from tableted dental fillings after 48 h. Maximum, and also comparable to the concentration of zinc ions was obtained with formulation Thymodentin (55.61 µg/mL) and self-hardening paste Coltosol F (10.36 µg/L). The lowest concentration was determined in a solution made up from tablets from a mixture formulation Thymodentin and water (0.4 µg/mL).

For the determination of the concentration of zinc ions in the artificial saliva solution after 168 h, the highest indicated concentration in the supernatant solution was obtained for the tablets made from the Thymodentin (73.04 µg/mL). As in the case of the concentration at 2 and 48 h, high value of Zn ion concentration was also obtained for the paste Coltosol F. For dental fillings made from Thymodentin and water, the concentration of zinc ions was almost 36 times lower than for tablets made only from Thymodentin preparation.

The daily requirement for zinc ions is about 15 mg and the potential toxic effect is observed after 1 g of zinc supplementation adopted for a long time (which could be observed in the case of dental fillings, usually applied through time over two weeks).

CONCLUSION

Tableting process is suitable for manufacturing temporary solid dental fillings, that may be used in dental application, after wet granulation. Suitable physical parameters provide solid dental filling useful in dental practice. Additionally, defined content of zinc ions allows to determine the volume of eugenol that should be used to ex tempore preparation of solid dental fillings that cause the repeatability of amount of zinc oxide and eugenol applied into tooth. In proposed method, differences and variability appearing from experience of person preparing temporary solid dental filling are going to be eliminated. Also, taking into consideration non unified shape of ready dental fillings in a tooth, tablets of a bound temporary dental filling mixture were used for its qualitative and quantitative composition.

REFERENCES

10. Modena K.C.S., Casas-Apayco L.C., Atta M.T.,
12. Pinto K.T., Stanislawczuk R., Loguerco A.D.,
13. Madarati A., Rekab M.S., Watts D.C.,
14. Ido T., Kumakiri M., Kiyohara T., Sawai T.,
Hasegawa Y.: Contact Dermatitis 47, 51 (2002).
15. Yanagi T., Shimizu T., Abe R., Shimizu H.:
17. Opoka W., Adamek D., Płonka M., Reczyński
W., Baś B. et al.: J. Physiol. Pharmacol. 61, 581
(2010).
22. Opoka W., Jakubowska M., Baś B., Sowa-
(2011).
23. Opoka W., Szlósarczyk M., Maślanka A., Piech
R., Baś B. et al.: Acta Pol. Pharm. Drug Res. 70,
961 (2013).

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Post-formulation studies of a product mainly involve its in vitro dissolution analysis to assess its safety and effectiveness in living body (1). In contrast to complex and lengthy in vivo testing, the in vitro dissolution analysis is an important quality control test, which simulates the possible in vivo performance of dosage form. This test guides the formulator to improve the product’s quality through modification of various variables. These variables are related to the process and/or formulation strategies (2). Process related variables include formulation development technique, stirring speed, order and speed of mixing, and effect of temperature. On the other hand, amount of drug, polymer, and/or excipients constitute the formulation variables. These variables can be varied to produce safe and effective formulation. Since it is not possible to assess these frequent changes through in vivo tests, there is significant dependence on in vitro dissolution analysis to approximate the probable in vivo outcome (3).

As one step advance, dissolution profiles obtained from these in vitro dissolution tests can be mathematically treated to predict plasma drug concentrations. One of the well-known mathematical approaches involves the use of convolution method. It is a direct method to predict the blood drug amounts from the dissolution profiles (4). Then, the predicted plasma drug concentration versus time data can be employed to evaluate three general pharmacokinetic parameters including C_{max} (maximum blood drug level), T_{max} (time required to attain maximum blood drug level), and AUC (area under blood drug concentration curve) followed by study of bioavailability and/or bioequivalence (4).

Thus, the aim of this article is to propose the effectiveness of convolution approach to predict...
pharmacokinetics of tramadol hydrochloride floating tablets.

**EXPERIMENTAL**

**Materials**

A gift sample of tramadol hydrochloride was got from Leo Pharmaceuticals, India. SD Fine Chemicals provided with carbopol and HPMC K100M. *Hibiscus rosa Sinensis* was supplied by Colorcon, India. All chemicals were analytical in quality and used without further purification.

**Preparation and in vitro evaluation of tablets**

The preparation and in vitro analysis of tramadol hydrochloride floating tablets is already reported (5). The sustained release tablets of tramadol hydrochloride were prepared using direct compression technique employing various ratios of carbopol, HPMC K100M, and *Hibiscus rosa Sinensis* as excipients and drug release controlling ingredients. Except magnesium stearate, all ingredients were weighed, sieved and mixed for 20 min to ensure uniform mixing. Before tableting, magnesium stearate was added and mixed for 30 s to ensure lubrication of mixture. For compression of mixture into tablets, single punch tablet machine having 8 mm flat-surface punches was used. The hardness of the prepared tablets was 4-6 kg/cm². For buoyancy, gas generation was achieved by sodium bicarbonate. The prepared tablets were named as F1 to F6, each contained 100 mg of tramadol. These tablets were then analyzed for quality control purpose adopting various tests including tablet dimensions, weight variation, friability, swellability, buoyancy, and drug content tests. The in vitro dissolution test was conducted using paddle method in 900 mL of HCl buffer with pH 1.2 to simulate the gastric condition. The stirring speed of paddles was set at 70 rpm. Temperature of dissolution medium was adjusted at 37 ± 3°C. At predetermined time points (0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 16, and 20 h), 5 mL of dissolved samples were taken with a replacement of the same volume using fresh medium. The obtained samples were analyzed at 271 nm using a UV visible spectrophotometer (Shimadzu, Japan). Dissolution data was passed through kinetic analysis using various kinetic models, i.e., zero-order, first-order, Higuchi and Korsmeyer-Peppas models to confirm the in vitro drug release fashion from the prepared tablets. The values of determination coefficient (R²) for zero-order, first-order, Higuchi and Korsmeyer-Peppas models ranged between 0.892-0.935, 0.894-0.965 and 0.822-0.895, respectively. From kinetic analysis, it was observed that first order equation gave the best fit to the dissolution data. From results, it is clearly evident that the prepared tablets showed the controlled release behavior through hydrodynamic balance, elaborating their gastro-retentive feature. The physicochemical properties for all tablets were within compendial limits (5, 6). The intactness of tramadol in formulation was confirmed by FTIR test (5). Each experiment was run in triplicate.

**Performance of convolution based calculations**

Convolution approach is useful to derive blood drug levels from in vitro dissolution data. This approach involves unit impulse response (Cₜ) and drug input rate in vitro (X′ₜₗₚₙ) as represented in following equation:

\[ c(t) = \int \delta(t-u) X'_{\text{in} \_ \text{vitro}}(u)du \]  

(Equation 1)

where, cₜ is typically assessed from intravenous bolus dose data or standard oral solution data. It represents drug concentration time data resulting from the instantaneous absorption of a particular drug amount. Moreover, the function c(t) corresponds to the plasma drug level versus time of the prepared formulations. In addition, X′ₜₗₚₙ and u represent the drug input rate of the oral solid formulation and variable of integration, respectively (4).

For determining blood drug levels (Cₜₘₚ) from the in vitro dissolution profiles, the listed steps were followed: (i) the percentage values of in vitro dissolution data were changed into the respective discrete amounts of drug released from formulation during each sampling interval; (ii) these discrete amounts of drug released were changed into the bioavailable amounts of drug using drug’s bioavailability data taken from a published article (7). This previously published article reported the values for half life, elimination rate constant (Kₑ), bioavailability factor, volume of distribution (Vₐ), body weight, and dose as 6.4 h, 0.111 1/h, 0.7, 5.15 L/kg, 63.8 kg, and 100 mg, respectively; (iii) the declining Cₜₘₚ during each interval [([Cₜₘₚ])] were determined using Kₑ value, adapted from previous publication (7); (iv) all (Cₜₘₚ) values for each time point were added to get final blood drug quantities; and (v) these quantities at each time point were divided by its Vₐ, adapted from previous publication (7), to Cₜₘₚ. This outcome acts as predicted blood drug profile, from which different pharmacokinetic parameters can be calculated.

**Data analysis**

Microsoft Excel 2007 was used to analyze data. The results are presented as the mean ± standard deviation.
RESULTS AND DISCUSSION

The *in vitro* dissolution profiles and predicted blood drug levels of F1-F6 tablets are presented in Figures 1 and 2, respectively. At last, predicted $D_0$ data were utilized to determine pharmacokinetic parameters including AUC, $C_{\text{max}}$ and $T_{\text{max}}$ using one compartment model approach (Table 1). For comparing the *in vitro* and *in vivo* profiles, $C_0$ are not compared due to the tremendous inter- and intra-subject variability. To get rid of this problem, the observed and the predicted pharmacokinetic parameters including $C_{\text{max}}$ and AUC are compared. The predicted values of $C_{\text{max}}$ (ng/mL) for F1, F2, F3, F4, F5, and F6 were 119.6 ± 4.7, 114.2 ± 7.1, 113.4 ± 5.9, 101.1 ± 5.8, 80.8 ± 3.2 and 84.1 ± 4.9, respectively. The predicted values of $T_{\text{max}}$ (h) were 12.1 ± 0.3, 12.2 ± 0.2, 12.2 ± 0.2, 11.4 ± 0.2, 11.7 ± 0.3 and
11.6 ± 0.3, respectively. The predicted values of AUC (ng.h/mL) were 1970.6 ± 287.4, 1921.7 ± 260.1, 1883.3 ± 301.7, 1589.1 ± 293.0, 1449.2 ± 240.1, and 1430.5 ± 209.5, respectively. These predicted values for AUC and Cmax are comparable to those previously reported observed values. The reported observed values for AUC and Cmax are 1227.3 ± 537.4 ng.h/mL and 217.8 ± 61.9 ng/mL, respectively (8). This similarity between published and our predicted results validates the application of convolution approach. The difference in dissolution features for F1-F6 products are noticeably responsible for difference in their respective predicted CB, which further leads to difference in their AUC and Cmax. It again validates the convolution approach.

The convolution approach (equation 1) has directly been applied to predict blood drug levels from in vitro dissolution data (4). After formulating dosage forms (F1-F6) with particular release characteristics, the dosage forms were assessed for their in vitro release feature aiming at the prediction of in vivo drug release in normal gastric physiology. The in vitro dissolution profiles and predicted blood drug levels of F1-F6 are presented in Figures 1 and 2, respectively. At last, predicted Dp data were utilized to determine pharmacokinetic parameters including AUC, Cmax, and Tmax (Table 1).

On the basis of Cmax and AUC, there are various approaches available in the literature that deal with the prediction of Cb from in vitro dissolution study (4). These approaches, however, need complicated approximating procedures, sometime with help of special software. In addition to these methods, convolution based approach appears to provide a good information about drug product’s quality by using basic knowledge of pharmacokinetics and a simple spread-sheet software. This method has been discussed in detail in this article starting from in vitro dissolution data to gaining of pharmacokinetic parameters. In addition, convolution method of predicting Cb is not product-specific, i.e., it does not require any in vivo data of that specific formulation, rather it is a guiding marker used to get needed in vivo profile using only available in vitro findings.

This method of comparing the observed and the predicted blood drug levels assumes similarity between in vitro and in vivo systems. But actually, there is wide diversity between the in vivo and the in vitro conditions. It includes variation in vessel sizes, dissolution medium volumes as well as the mixing rate. The in vivo drug dissolution and the absorption is affected by numerous physiological factors, consequently pharmacokinetics is tremendously erratic also. On the contrary, such inconsistency is not
imagined for in vitro data, because the in vitro dissolution testing is characteristically conducted under virtually controlled homogenous circumstances (4). This approach is equally effective for assessing the bioavailability/bioequivalence. After adopting some change in manufacturing, dissolution analysis of new formulation can be used to approximate its Cth, followed by their evaluation in accordance with criteria for bioavailability/bioequivalence. If the profiles congregate the bioequivalence criteria then it should be believed that the manufacturing modifications had no adverse influence on the product quality. Otherwise, formulation development would need alteration consequently (9).

CONCLUSION

To compute blood drug levels from in vitro dissolution data, the convolution approach is a useful mathematical procedure which is free from complicated and lengthy in vivo study procedures. However, freedom from physiological variabilities that affect observed blood drug levels is a limitation of this modality. However, this limitation can be minimized by using biosimilar dissolution test for improved prediction of blood drug concentration.

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REFERENCES


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Inflammation is a response of the immune system to physical and/or chemical and/or biological injury, defined as any process able to cause tissue or cell damage. Inflammatory processes are cause of a large number of diseases, such as atherosclerosis, cancer, asthma, arthritis, and many others (1).

Theophylline (Th) - 1,3-dimethyl-3,7-dihydro-purine-2,6-dione has been widely used for the treatment of airway diseases for more than 80 years (2). More recently, it has been shown to have anti-inflammatory effects in asthma and chronic obstructive pulmonary disease (COPD) at lower concentrations. The molecular mechanism of the anti-inflammatory effect may be due to inhibition of PDE4 and histone deacetylase-2 activation, resulting in switching off activated inflammatory genes (3). Theophylline has a narrow therapeutic index; as a result, toxicity can be a significant problem with its chronic use.

This is the reason why new, more efficient and free of side effects anti-inflammatory medications are constantly searched for. The modification of a parent structure of theophylline by introduction of 4-arylpiperazinyl-alkyl substituent in the 7 position allowed to receive potent analgesic, antipyretic, and antiphlogistic agents in the group, the most significant activity in several in vivo models (e.g., acetic acid writhing test, bradykinin-induced pain response, carrageenan-induced paw edema) was observed for compounds possessing 3 ñ 5 carbon alkyl chain and substituted phenyl ring with electron-withdrawing chloro/fluoro atom and methyl or trifluoromethyl group (4).

In the context of a research program that aims to contribute to the discovery of new anti-inflammatory and analgesic drug candidates, we described the synthesis and pharmacological evaluation of new 8-
alkoxy-1,3-dimethyl-2,6-dioxopurin-7-yl derivatives with ester (1, 2), carboxylic (3, 4) and amide (5, 6, 8–11) terminal groups (5).

We also tested 8-oxo-purine-2,6-dione analogue (7) with an additional acid function in the form of an enol group (Fig. 1) (5).

The tested compounds 1–11 showed analgesic activity. The strongest analgesic and anti-inflammatory effects were observed for benzylamide (6) or 4-arylpiperazinamide (8–11) derivatives, which were more active than acetylic acid used as a reference drug (up to 23- and 36-fold increase in activity in writhing and formalin test, respectively). Several compounds more active than theophylline inhibited the phosphodiesterase activity in rat liver homogenates (5).

In the present paper, the results of further pharmacological studies in a group of 8-alkoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl derivatives (1–11) are described. They concern the evaluation of anti-inflammatory activity of these compounds in the zymosan-induced peritonitis model and the carrageenan-induced hind paw edema model in mice. We also assessed their activity in in vitro FRAP assay estimating the total ferric reducing antioxidant power. Finally, the effect of the most active theophylline derivatives on plasma TNF-α level in rat model of endotoxemia was investigated.

EXPERIMENTAL

Chemistry

The multistep syntheses of the investigated compounds 1–11 (Fig. 1) were previously reported (5). Firstly, in a reaction of 8-bromo-1,3-dimethyl-1H-purine-2,6,8-trioxo-2,3,8,9-tetrahydro-1H-purin-7(6H)-ylacetate (7) was synthesized by acidic hydrolysis of 1 following by estification with methanol. Benzylamides (5, 6) and arylpiperazinamides (8–11) were prepared in a reaction of 3 and 4 with respective amine (benzylamine or 1-arylpiperazine derivatives), using 1,1′-carbonylimidazole (CDI) as carbonyl group activating agent in DMF medium (5). The chemical structure of compounds 1–11 were confirmed by spectral data (1H-NMR, LC/MS) and elemental analyses and the purity were establish using LC/MS method. All the investigated compounds have purity over 98% (5).

Pharmacology

Animals

Experiments were carried out on male Wistar rats weighing 180-220 g and male albino Swiss mice (18–26 g). The animals were housed in constant temperature facilities exposed to 12 : 12 light-dark cycle and maintained on a standard pellet diet and tap water given ad libitum. Control and experimental groups consisted of 6–8 animals each. The investigated compounds were administered intraperitoneally (i.p.) in a form of suspension in 0.5% methylcellulose. Control animals received the equivalent volume of solvent.

Male Wistar rats weighing 200-250 g bred in-house from progenitors obtained from Charles River Laboratories (Sulzfed, Germany) were used to assess the influence of theophylline derivatives on plasma TNF-α levels in a model of endotoxemia. Animals were fasted overnight prior to drug administration but had free access to water.

All procedures were conducted according to guidelines of ICLAS (International Council on Laboratory Animals Science) and were approved by The Local Ethics Committee of the Jagiellonian University in Kraków (agreement nr 47/2014).
Drugs and chemicals

LPS (Escherichia coli 055:B5), methylcellulose, carrageenan, zymosan A, Evans blue, ketoprofen, indomethacin, acetate buffer, FeCl₃·6H₂O, TPTZ (2,4,6-tripyridyl-s-triazine), theophylline, ascorbic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Carrageenan-induced edema model

Wistar rats weighing 180-220 g were used in the “hind paw edema” test. Animals were divided into four groups, one of them being the control. In order to produce inflammation, 0.1 mL of 1% carrageenan solution in water was injected into the hind paw subplantar tissue of rats, according to the modified method of C.A. Winter (6) and P. Lence (7). The development of paw edema was measured with a plethysmometer (Plethysmometer 7140, Ugo Basile). Prior to the administration of test substances, paw diameters were measured by dividers and recorded. The investigated compounds were administered at doses of 5, 10, 25, 50, 100 mg/kg, i.p. (as a suspension in methylcellulose), prior to carrageenan injection. Methylcellulose was administered by the same route, to the control group (methylcellulose had no effect on edema, data not shown). After these administrations, paw diameters were measured at 1, 2 and 3 h. The percent, of edema and edema inhibition were calculated according to the equations given below.

\[
\text{Edema \%} = \left( \frac{N - N'}{N} \times 100 \right) / N
\]

\[
\text{Edema inhibition \%} = \left( \frac{N - N' \times 100}{N} \right) / N
\]

where \(N\) = paw diameters measured 1, 2 and 3 h after injection of carrageenan to the control group; \(N'\) = paw diameters measured 1, 2, and 3 h after injection of carrageenan to the test groups; \(N'\) = paw diameters at the beginning.

Zymosan-induced peritonitis

Peritoneal inflammation was induced as described previously (8). Zymosan A was freshly prepared (2 mg/mL) in sterile 0.9% NaCl. Thirty min after subcutaneous (s.c.) injection of the investigated compounds into the loose skin over the flank, zymosan A was injected i.p. in a volume of 0.25 mL. Four hours later, the animals were killed. The peritoneal cavity was lavaged with 1.5 mL of saline and after 30 s of gentle manual massage the exudates were retrieved. Cells were counted using an automatic cell counter (Countess, Invitrogen) following staining with Turk’s solution. The investigated compounds suspended in 0.5% methylcellulose were injected s.c. at the dose of 50 mg/kg b.w. and pitched in an ultrasonic cleaner. The control group was given s.c. 0.5% methylcellulose 30 min prior to zymosan.

Vascular permeability

The compounds suspended in 0.5% methylcellulose were injected s.c. at the dose of 50 mg/kg b.w. Then, after 30 min, Evans blue was suspended in saline (10 mg/mL) and injected intravenously (i.v.) into the caudal vein (0.2 mL/mouse), which was immediately followed by i.p. injection of zymosan A. Thirty minutes later the animals were killed and their peritoneal cavities were lavaged with 1.5 mL of saline as described above. The lavage fluid was centrifuged and the absorbance of the supernatant was measured at 620 nm as described previously (9). The investigated compounds suspended in 0.5% methylcellulose were injected s.c. 30 min before Evans blue and zymosan. The control group was given s.c. 0.5% methylcellulose 30 min prior to zymosan. Indomethacin in a dose of 50 mg/kg b.w. was used as a reference compound.

Determination of the antioxidant activity by the FRAP assay

The FRAP assay was conducted according to Benzie and Strain (10) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g C₂H₃NaO₂·3H₂O and 16 mL C₂H₄O₂), pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM FeCl₃·6H₂O solution was prepared by mixing 10 parts of acetate buffer, 1 part of TPTZ solution, and 1 part of FeCl₃·6H₂O solution. Three hundred \(\mu\)L of the FRAP solution was mixed with 10 \(\mu\)L of the test compound solution and incubated at room temperature for 10 min in the dark. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm against ethanol. The results for the test compounds are expressed as an increase in absorbance of the test sample compared to a sample containing the solvent.

In the FRAP assay the antioxidant potential of the sample was determined from a standard curve plotted using FeSO₄·7H₂O in a concentration range between 37.5 and 1200 \(\mu\)M.

LPS-induced endotoxemia

Rats were cannulated in the jugular vein under ketamine/xylazine anesthesia three days before the experiment. All catheters were filled with heparinized saline and exteriorized via an incision on the back of the neck. In order to induce endotoxemia, an intravenous dose of 1 mg/kg lipopolysaccharide (LPS) from E. coli serotype 055:B5 was adminis-
tered prior to compound administration. Compounds 1, 3, 4, 6, 7, 9 and theophylline (Th) as a reference drug suspended in 0.5% methylcellulose were given to rats ($n = 4-5$) at a dose of 50 mg/kg i.p. simultaneously with LPS. Control animals received LPS and a respective volume of vehicle by the same routes of administration as the treatment groups. Blood samples (300 µL) were collected into heparinized tubes at 0, 15, 30 min and 1, 1.5, 2, 3, and 4 h after LPS and compound administration. The animals were injected with an equal volume of 0.9% saline through the tubings after each blood collection. Blood was centrifuged at 4°C for 20 min (1500 × g). Plasma was stored at −80°C until assayed.

**Determination of plasma TNF-α levels**

Tumor necrosis factor α (TNF-α) concentrations in rat plasma were measured using ELISA (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Limit of quantification was 13 pg/mL.

**Statistical analysis**

The data are expressed as the mean ± SEM (standard error of the mean). To compare the results between two different groups of animals (the investigated compound group vs. the control group) Student’s $t$-test was used. The difference of means was statistically significant if $p < 0.05$. The results of carrageenan-induced paw edema experiments are expressed as a percentage of change from control (pre-drug) values. The data were evaluated by one-way analysis of variance (ANOVA) followed by Duncan’s test. A probability value $< 0.05$ was considered statistically significant.

| Table 1. Antiinflammatory effect of the compounds in the carrageenan-induced paw edema test. |
|----------------------------------|-----------------|-----------------|-----------------|-----------------|
| **Compound** | **Dose mg/kg** | **1 h** | **2 h** | **3 h** |
| Control | - | 0.9 ± 0.01 | 1.35 ± 0.01 | 1.52 ± 0.09 |
| 1 | 100 | 0.5 ± 0.09 | 0.25 ± 0.08** | 0.030 ± 0.004*** |
| | 50 | 0.65 ± 0.07 | 0.35 ± 0.05* | 0.25 ± 0.03** |
| 2 | 100 | 0.25 ± 0.03* | 0.23 ± 0.04** | 0.18 ± 0.02** |
| | 50 | 0.73 ± 0.08 | 0.35 ± 0.06* | 0.25 ± 0.08* |
| 3 | 100 | 0.6 ± 0.07 | 0.08 ± 0.006*** | 0.020 ± 0.001*** |
| | 50 | 0.75 ± 0.09 | 0.3 ± 0.07* | 0.18 ± 0.02** |
| 4 | 100 | 0.6 ± 0.07 | 0.38 ± 0.09* | 0.050 ± 0.004*** |
| | 50 | 0.6 ± 0.04 | 0.325 ± 0.02* | 0.25 ± 0.03** |
| 5 | 100 | 0.5 ± 0.03 | 0.080 ± 0.007*** | 0.020 ± 0.001*** |
| | 50 | 0.6 ± 0.08 | 0.375 ± 0.09* | 0.28 ± 0.02** |
| Control | - | 0.9 ± 0.13 | 1.04 ± 0.1 | 1.22 ± 0.09 |
| 6 | 25 | 0.075 ± 0.005*** | 0.175 ± 0.01*** | 0.1 ± 0.07*** |
| | 10 | 0.475 ± 0.09* | 0.175 ± 0.01*** | 0.1 ± 0.07*** |
| 7 | 100 | 0.52 ± 0.08 | 0.21 ± 0.05** | 0.030 ± 0.005*** |
| | 50 | 0.61 ± 0.04 | 0.37 ± 0.05* | 0.22 ± 0.03** |
| 8 | 25 | 0.1 ± 0.02*** | 0.01 ± 0.002*** | 0.075 ± 0.005*** |
| | 10 | 0.175 ± 0.03** | 0.125 ± 0.009*** | 0.15 ± 0.009*** |
| 9 | 25 | 0.2 ± 0.04*** | 0.075 ± 0.008*** | 0.125 ± 0.02*** |
| | 10 | 0.225 ± 0.01** | 0.325 ± 0.02** | 0.15 ± 0.009*** |
| 10 | 25 | 0.175 ± 0.03** | 0.125 ± 0.04*** | 0.1 ± 0.006*** |
| | 10 | 0.65 ± 0.1 | 0.35 ± 0.07** | 0.15 ± 0.03*** |
| 11 | 25 | 0.225 ± 0.05** | 0.175 ± 0.01*** | 0.075 ± 0.003*** |
| | 10 | 0.2 ± 0.06** | 0.125 ± 0.009*** | 0.15 ± 0.005*** |

Data are presented as the means ± SEM of 6–8 animals per group. The results were analyzed by one-way analysis of variance (ANOVA)

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control value at respective time points.
Comparisons between maximum TNF-α levels and the area under effect-time curve (AUEC) were performed using a one-way ANOVA with post-hoc Tukey HSD test (Statistica v. 10, StatSoft, USA). AUEC was calculated using Phoenix WinNonlin v. 6.3 (Pharsight Corporation, Mountain View, CA, USA).

RESULTS

Anti-inflammatory (antiedematous) effect in the carrageenan-induced edema model

All new 8-alkoxy-1,3-dimethyl-2,6-dioxopurin-7-yl derivatives were first administered at a dose of 10 mg/kg. In the case of the compounds that were not active at this dose, in the next step, they were administered at higher doses, while others at a lower dose. Therefore, the compounds 1–5 and 7 were administered at doses from 100 to 10 mg/kg body weight, whereas compounds 6 and 8–11 at doses from 25 to 5 mg/kg b.w. Ketoprofen was used as a reference compound. Ketoprofen administered i.p. at a dose of 100 mg/kg b.w. inhibited edema formation by 80.0%, 75.7%, and 80.6%, in three consecutive hours of the experiment, respectively. When administered i.p. at a dose of 20 mg/kg b.w. it inhibited edema formation by 20, 54 and 67% in three consecutive hours of the experiment, respectively. All test compounds decreased the volume of edema induced by s.c. carrageenan injection into the hind paw of rats (Table 1).

A statistically significant antiedematous effect was observed after the administration of compound 1 at two doses. The doses of 100 mg/kg b.w. and 50 mg/kg b.w. reduced edema by 81.4-98.0% and 74.0-83.5% in the 2nd and 3rd hour of the experiment, respectively. Its dose of 10 mg/kg b.w. reduced edema by 72.2 and 81.5% (Table 1).

Compound 6 also exhibited a statistically significant effect in this experimental model. When administered i.p. at a dose of 25 mg/kg b.w., it inhibited edema formation by 91.6, 83.2, and 91.8% in the 1st, 2nd and 3rd hour of the experiment, respectively. Its dose of 10 mg/kg b.w. reduced edema by 47.2, 83.2, and 91.8%. A statistically significant antiedematous effect was observed after the administration of compound 7 at two doses. Doses of 100 mg/kg b.w. and 50 mg/kg b.w. reduced edema by 79.8-64.4% and 97.5-81.9% in the 2nd and 3rd hour of observation, respectively. Compound 8 when administered at doses 25 mg/kg b.w. and 10 mg/kg b.w. significantly lowered edema throughout the whole observation period by 88.8-93.8% and 80.5-87.7%, respectively.

Compound 9 showed anti-inflammatory effect in a dose range from 5 mg/kg b.w. to 25 mg/kg b.w. but only two highest doses significantly reduced edema formation by 68.7-92.7% (Table 1). The theophylline derivative 10 at a dose of 25 mg/kg b.w. produced a strong anti-inflammatory effect inhibiting edema of the mouse hind paw by 80.5, 88 and 91.8% in the 1st, 2nd and 3rd hour after carrageenan injection, respectively. When the dose was lowered, the activity of this compound slightly decreased. Compound 11 administered at doses of 25 mg/kg b.w. and 10 mg/kg b.w. demonstrated a significant anti-inflammatory effect. This effect increased with elapsing time of the experiment reaching the maximum in the 3rd hour. Dose lowering caused extinction of the anti-inflammatory activity.

The effects of the compounds on vascular permeability during zymosan-induced peritonitis

The effect of the investigated compounds on vascular permeability was tested at the dose of 50 mg/kg b.w. Indomethacin at the dose of 50 mg/kg b.w. was used as a reference compound. The intensity of early vascular permeability was significantly inhibited in the groups receiving compounds 1, 3 and 4 compared to the control group (Table 2). These compounds decreased the vascular permeability by 94.3-81.2%. The reducing effect on the vascular permeability of these compounds was greater.
than that of indomethacin. The early vascular permeability was significantly inhibited also in the groups receiving compounds 5-11 compared to the control group (Table 2). These compounds decreased the vascular permeability by 77.3 to 61.1%. In turn, compound 2 influenced vascular permeability during zymosan-induced peritonitis, but the effect was not statistically significant.

The effects of the compounds on infiltration of neutrophils during zymosan-induced peritonitis

The effect of the investigated compounds on infiltration of neutrophils was tested at the dose of 50 mg/kg b.w. The early infiltration of neutrophils measured at 4 hours following zymosan injection was significantly stronger than that of indomethacin and was inhibited in the group receiving compound

Table 2. Percent inhibition of vascular permeability in zymosan-induced peritonitis in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose [mg/kg]</th>
<th>Absorbance</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>2.230 ± 0.180</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>0.138 ± 0.050</td>
<td>93.8***</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>1.400 ± 0.190</td>
<td>37.2</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0.126 ± 0.070</td>
<td>94.3***</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>0.418 ± 0.040</td>
<td>81.2**</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>0.739 ± 0.021</td>
<td>66.8*</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>0.728 ± 0.028</td>
<td>67.3*</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>0.700 ± 0.030</td>
<td>68.6*</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>0.825 ± 0.039</td>
<td>63.0*</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>0.588 ± 0.060</td>
<td>77.3**</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>0.798 ± 0.046</td>
<td>64.2*</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>0.867 ± 0.063</td>
<td>61.1*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>50</td>
<td>0.456 ± 0.120</td>
<td>79.5**</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student t-test, * p < 0.05,** p < 0.01,*** p < 0.001 vs. control.

Table 3. Percent inhibition of neutrophil infiltration in zymosan-induced peritonitis in mice.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose [mg/kg]</th>
<th>Count of neutrophils</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>6.4 × 10^5 ± 0.76</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>50</td>
<td>8.3 × 10^4 ± 0.52</td>
<td>87.0**</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>4.4 × 10^5 ± 1.36</td>
<td>31.2</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>2.1 × 10^6 ± 0.18</td>
<td>96.7***</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>8.4 × 10^5 ± 0.28</td>
<td>86.8**</td>
</tr>
<tr>
<td>5</td>
<td>50</td>
<td>2.1 × 10^6 ± 0.16</td>
<td>67.1*</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>1.9 × 10^6 ± 0.26</td>
<td>70.3*</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>2.4 × 10^6 ± 0.15</td>
<td>62.5*</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>2.9 × 10^6 ± 0.62</td>
<td>54.6*</td>
</tr>
<tr>
<td>9</td>
<td>50</td>
<td>1.7 × 10^6 ± 0.85</td>
<td>73.4*</td>
</tr>
<tr>
<td>10</td>
<td>50</td>
<td>2.6 × 10^6 ± 0.74</td>
<td>59.3*</td>
</tr>
<tr>
<td>11</td>
<td>50</td>
<td>1.9 × 10^6 ± 0.62</td>
<td>70.3*</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>50</td>
<td>9.8 × 10^5 ± 0.29</td>
<td>84.6**</td>
</tr>
</tbody>
</table>

Data are presented as the means ± SEM of 6–8 mice per group. The results were analyzed by Student t-test, * p < 0.05,** p < 0.01,*** p < 0.001 vs. control.
I (decrease by 87.0%) and in the group treated with 3 (decrease by 96.7%), and 4 (decrease by 86.8%).

The early infiltration of neutrophils was significantly inhibited also in the groups receiving compounds 5-11 compared to the control group. These compounds decreased the count of neutrophils by 73.4 to 59.3% (Table 3). Compound 2 influenced infiltration of neutrophils during zymosan-induced peritonitis, but the effect was statistically non-significant.

**Antioxidant activity measured by the FRAP assay**

Investigation of the effect of the test compounds on the total antioxidant power revealed that 9 of them: 1, 2, 4 and 6-11 at a concentration of 10^{-5} M increased the total ferric reducing antioxidant ability by 0.29-3.7% of the maximum value obtained for ascorbic acid at the same concentration. The remaining compounds did not show an antioxidant effect in this test.

**Inhibition of TNF-α production in vivo**

Administration of compounds 1, 3, 4, 6, 7, and 9 to rats with LPS-induced endotoxemia caused a significant inhibition of TNF-α production in plasma (Fig. 2). The most pronounced effect was observed in the presence of compounds 3 and 1. The maximum concentration of TNF-α achieved at 1.5 h following LPS administration decreased significantly from 20157 pg/mL (LPS + vehicle) to 2579 and 3269 pg/mL, respectively (Fig. 3). At the same time, compounds 4 and 9 decreased the maximum concentration of TNF-α by 75.9 and 71.8%, respectively, and these differences were also statistically significant. The weakest inhibitory effect exhibited compounds 6 and 7. For these compounds TNF-α cytokine concentrations at 1.5 h were more than 3 times higher when compared with the strongest inhibitor of TNF-α that is compound 3 (a decrease by 57.6 and 55.7% compared to the control group, i.e., LPS + vehicle). In addition, these concentrations were 1.5 times higher than that observed following...
Th administration (5665 pg/mL) used as a reference compound in this study. Similar differences were observed between the areas under effect curve (AUEC) calculated based on the individual TNF-α time profiles in the vehicle group and all study groups (Fig. 3).

DISCUSSION AND CONCLUSION

The aim of the study was to evaluate of anti-inflammatory activity of new methylxanthine derivatives. For this purpose we used zymosan-induced peritonitis and the carrageenan-induced hind paw edema models in animals (11). The carrageenan test is used to evaluate the anti-inflammatory effect; injection of this compound into the hind paw of an animal induces a long-lasting edema (12, 13).

The carrageenan-induced paw edema model demonstrated that compounds 6 and 8-11 containing amide substituent showed the greatest anti-inflammatory (antiedematous) effect. The effect of all these compounds was comparable and increased with time elapsing from the beginning of the experiment reaching the maximum in the 3rd hour. The effect was dose-dependent. The above compounds produced the highest statistically significant effect at the dose of 25 mg/kg b.w. All these methylxanthine derivatives revealed a stronger action compared with ketoprofen. The administration of a lower dose of 10 mg/kg b.w. only slightly diminished anti-inflammatory activity.

The remaining compounds showed also anti-inflammatory activity but at higher doses as they were administered at 100-10 mg/kg b.w. in this test. A statistically significant effect was observed (except for compound 2) only in the 2nd and 3rd hour of the experiment. The administration of a lower dose of 50 mg/kg b.w. induced only a minute lowering of anti-inflammatory activity.

Murine zymosan-induced peritonitis was described as a suitable model of acute inflammation, characterized by vascular changes and production of inflammatory mediators leading to leukocyte accumulation in the inflammatory focus (14, 15). Two major events are critical for development of zymosan-induced peritonitis, namely the early increase in vascular permeability (< 1 h) and the infiltration of neutrophils into the peritoneum that follows after some hours (9). The mechanisms operating during the above stages have been investigated and these studies revealed that early vascular permeability depends mostly on cysteinyl-leukotrienes released by resident peritoneal macrophages and, to lesser extent, on mast cell histamine and prostaglandins (PGE₂, prostacyclin) of multiple cellular origins (9).

This study focused on two major events leading to the development of inflammation, i.e., the early increase in vascular permeability and neutrophil infiltration into peritoneum. The results indicated that the compounds under investigation demonstrated anti-inflammatory activity in both tests. In the study of cellular infiltration they significantly limited the migration of leukocytes to site of inflammation, which was the peritoneum.

Compounds 1 (with ester terminal moiety) as well as 3 and 4 (containing carboxylic group) showed the strongest anti-inflammatory activity in the zymosan-induced peritonitis.

Compounds 3 and 4 with terminal carboxylic moiety significantly reduced the vascular permeability by 94.3 and 81.2% and inhibited infiltration of neutrophils by 96.7 and 86.8%, respectively, compared to the control group. In turn, 8-alkoxy-1,3-dimethyl-2,6-dioxopurine-7-yl derivative with ester terminal group, i.e., compound 1 reduced the vascular permeability by 93.8% and inhibited infiltration of neutrophils by 87.0%, while 8-oxo-purine-2,6-dione analogue (compound 7) reduced the vascular permeability by 68.6% and inhibited infiltration of neutrophils by 62.5%. The statistical analysis showed that compounds 6 and 8-11 (containing amide and benzylamide) significantly reduced the vascular permeability by 61.1-77.3% compared to the control group. The obtained data also demonstrated that administration of these compounds at the same dose (50 mg/kg b.w.) significantly inhibited infiltration of neutrophils by 54.6-73.4%.

Summing up, all compounds 1-11 showed statistically significant anti-inflammatory activity in both tests: the carrageenan-induced edema test and the zymosan-induced peritonitis (except for compound 2). They significantly reduced the early vascular permeability, inhibited infiltration of neutrophils and tempered paw edema formation. In the carrageenan-induced paw edema model, compounds 6 and 8-11 showed the greatest anti-inflammatory (antiedematous) effect. In the case of the zymosan-induced peritonitis, compounds 3 and 1 followed by 4 and 9 revealed the greatest anti-inflammatory activity.

The mechanism of tissue damage due to inflammatory processes has been partly linked to the release of reactive oxygen species (ROS) from activated neutrophils and macrophages. Excessive ROS production leads to tissue damage by degradation of macromolecules and peroxidation of membrane lipids. On the other hand, reactive oxygen species
support and spread inflammation by the stimulation of cytokine production (IL-1, TNF-α, INF-γ) which increase further neutrophil and macrophage influx (16). Thus, free radicals are indispensable mediators of inducing and maintaining inflammation while their neutralization by antioxidants and free radical scavengers can limit its severity (17).

In order to elucidate anti-inflammatory activity of theophylline derivatives, we determined the total ferric reducing antioxidant power (FRAP). The pharmacological studies demonstrated that the test compounds were practically almost completely devoid of antioxidant activity. Therefore, their anti-inflammatory activity does not result from the influence on the total antioxidant potential.

Due to the important role of TNF-α in inflammation, further attempts to clarify the mechanism of action of the most active test compounds involved determination of TNF-α levels after their administration to rats with LPS-induced endotoxemia.

TNF-α is one of the major proinflammatory cytokines that stimulates the release of other mediators of inflammation, thereby inciting further inflammatory responses (18). The TNF-α expression is mainly under the regulatory control of nuclear factor-κB (NF-κB).

LPS-induced models of sepsis and septic-shock are commonly used to evaluate efficacy of anti-inflammatory drugs (19). In vitro methods employing LPS-stimulated whole blood or murine macrophages, although less cost- and time-consuming, may provide results not entirely reflecting in vivo drug activity. It has been shown that IC50 values assessed in vitro were up to 10 times higher than those estimated in vivo (20). Thus, it seems that inhibition of TNF-α production following LPS administration to animals may be more appropriate to study the true efficacy of new compounds than in vitro methods.

The results of the in vivo experiment on a series of the investigated purine-2,6-dione derivatives in rats with endotoxemia indicate that all compounds studied significantly inhibited TNF-α production in rat plasma (Figs. 3 and 4). The results of the in vivo study indicate that among compounds studied 1 and 3 and also 4 and 9 are the strongest inhibitors of TNF-α production in rat plasma. For these compounds TNF-α concentrations were lower than that observed for theophylline used in this study as a reference compound. In addition, these compounds most strongly inhibited infiltration of neutrophils in the zymosan-induced peritonitis. Based on the results of preliminary study performed in our laboratory, compounds 1 and 3 are the weakest phos-

phodiesterase (PDE) inhibitors as assessed using rat liver homogenates (5). The strongest inhibition of PDE in the liver homogenates revealed compounds 6 and 7 (5). For these compounds TNF-α concentrations at 1.5 h were higher than that observed for theophylline.

This discrepancy between TNF-α and PDE-inhibition by studied compounds may be explained by differences in distribution pattern and relative abundance of PDEs in plasma and liver. For example, in CD4+ and CD8+ T-lymphocyte homogenates, substantial PDE 4 and PDE 3 and only low PDE 1, 2 and 5 activities were observed (21). Recently, it has been shown that PDE 4A, PDE 4B, PDE 4D and PDE 7A mRNA are present in similar quantities in both CD4+ and CD8+ lymphocytes (22). Monocytes exclusively contain PDE 4 but their in vitro matura-

tion leads to a PDE isozyme profile similar to that of alveolar macrophages (23). In turn, in the liver, PDE 2-4, 8, 9, and PDE 11 show relatively high expression, whereas PDE 7 is not detected.

Thus, TNF-α inhibition by new methylxantine derivatives observed in the study seem to be related to the increased levels of cAMP (an inhibitor of the NF-κB pathway) that is assumed to be the most important mechanism of action of most xanthines with anti-inflammatory activity (24, 25) or to a direct inhibition of NF-κB activation. The mecha-

nism of compounds in reducing inflammation is thought to be due to inhibition of TNF-α, a cytokine that has been shown to increase leukocyte adhesion and to disrupt intercellular junctions of postcapillary venular endothelium, leading to plasma extravasa-

tion. In the carrageenan-induced paw edema model, derivatives of theophylline reduced the edema response and in addition, inhibited infiltration of neutrophils in the zymosan-induced peritonitis. Probably the test compounds by reducing TNF-α act on endothelial cells inhibiting the expression of adhesion molecules and chemokines necessary for the accumulation of white blood cells at the site of inflammation.

In the next stage of research on these com-

pounds it is planned to test whether they have the ability to inhibit histone deacetylase, cyclooxygenase and Transient Receptor Potential (TRP) ankyrin 1 (TRPA1). Review of world literature provides data confirming that the anti-inflammatory effect of some drugs (including theophylline) results from the influence on histone deacetylase and ion channels TRPA1 (26, 27).

In summary, the study indicates that the 8-

alkoxy-1,3-dimethyl-2,6-dioxo-purin-7-yl deriva-

tives (1-11) are a new class of compounds with anti-

"Anti-inflammatory and antioxidant activity of..." 769
inflammatory activities. The carrageenan-induced paw edema model demonstrated that compounds 6 and 8-11 containing amide substituent showed the greatest anti-inflammatory (antiedematous) effect. The compounds with carboxylic (3, 4) and ester moiety (1) showed the highest anti-inflammatory activity in the zymosan-induced peritonitis. They significantly decreased neutrophils count and inhibited intensity of early vascular permeability. In addition, especially compound 3 and also compound 1 significantly inhibited of TNF-α production in plasma of rats with endotoxemia and this effect was stronger than that of theophylline. Compounds did not show significant antioxidant properties. Therefore, their beneficial effects observed in the models of inflammation used in this study are not related to the influence on the total antioxidant potential. The results obtained indicate that the mechanism of anti-inflammatory activity of compounds is probably related to the inhibition of TNF-α release.

REFERENCES


Received: 25. 03. 2015
Diabetes mellitus is one of the most common, non-communicable diseases worldwide. It is the main cause of heart diseases, stroke, kidney failure, lower limb amputations (non-traumatic) and new cause of blindness in adults and seventh main cause of deaths in the United States (1). The prevalence of diabetes is rapidly increasing especially in children and young adults. The number of type 2 diabetic patients is increasing worldwide continuously. Approximately, 80% of the diabetic people live in middle and low income countries of the world. Most of the diabetic patients were between 4-60 years of age. About 183 million diabetic patients were undiagnosed; 4.6 million deaths were caused by diabetes in 2011. Every year about 78000 children develop type 1 diabetes (2).

Today, hepatitis C is the most common blood born disease worldwide (3). Since the discovery of hepatitis C in 1989, it is the major cause of chronic liver disease and will be the substantial cause of mortality in the future. The complexity and uncertainty of hepatitis C relate to its geographical distribution and determination of its associated risk factors that accelerate progression of disease. In developed countries, it is the main cause of liver transplantation, and is the most common chronic blood born infection in the United States. The distribution of hepatitis C is quite variable geographically. The highest prevalence rates of hepatitis C have been reported in the countries of Africa and Asia. Lower prevalence rates have been recorded in the industrialized countries of North and Western Europe, North America and Australia (4-7).
Hepatitis C is both acute as well as chronic liver disease, most of the morbidity is associated with the development of chronic infection after initial acquisition. Determination of onset of HCV infection is difficult as most of the infections are asymptomatic initially (4).

The principal objective of this research is to elucidate relationship between diabetes mellitus and hepatitis C. Whether diabetes is a risk factor for HCV infection or not, taking into consideration the significance of other risk factors for hepatitis C in diabetic patients.

METHODOLOGY

For this descriptive cross-sectional study, non-probability purposive sampling technique was used. Sample size comprised of 100 diabetic patients as well as 100 healthy subjects (control group). Inclusion criteria comprised of both type 1 and 2 diabetic patients of any age, of either sex. Emergency cases, dialysis patients, transplant recipients and patients who didn’t give consent for participation were excluded from study.

Sample collection procedure

A detailed, structured questionnaire was developed to record demographic and clinical history of patients. Hundred diabetic patients who visited the diabetic clinic Nishtar Hospital Multan were interviewed. Informed consent of every participant was taken before sampling, ensuring them the confidentiality and describing them the objectives of the study. Patients were interviewed for demographic information, duration of diabetes, mode of therapy, hospital admission, intravenous drug addiction, family history of hepatitis C, nose or ear piercing, tattooing, tooth brush sharing, razor sharing, comb sharing and blood glucose level. Sample collection was carried out by using sterile disposable syringes (Becton Dickinson, Pakistan). Approximately 3 mL of peripheral blood of every participant (diabetic patient) was obtained via veinopuncture technique and labeled accordingly. Then, the samples were transferred to the laboratory for analysis. Blood samples of 100 healthy volunteers (control group) visiting blood bank of Nishtar Hospital, Multan were also taken and tested for anti-HCV antibodies.

<table>
<thead>
<tr>
<th>No. of obs.</th>
<th>Variables</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type 1 diabetes</td>
<td>15%</td>
</tr>
<tr>
<td>2</td>
<td>Type 2 diabetes</td>
<td>85%</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>35%</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>65%</td>
</tr>
<tr>
<td>5</td>
<td>Married</td>
<td>95%</td>
</tr>
<tr>
<td>6</td>
<td>Unmarried</td>
<td>5%</td>
</tr>
<tr>
<td>7</td>
<td>Urban</td>
<td>91%</td>
</tr>
<tr>
<td>8</td>
<td>Rural</td>
<td>9%</td>
</tr>
<tr>
<td>9</td>
<td>Blood transfusion</td>
<td>17%</td>
</tr>
<tr>
<td>10</td>
<td>Hospital admission</td>
<td>66%</td>
</tr>
<tr>
<td>11</td>
<td>Major surgery</td>
<td>48%</td>
</tr>
<tr>
<td>12</td>
<td>Family history of HCV inf.</td>
<td>21%</td>
</tr>
<tr>
<td>13</td>
<td>Toothbrush sharing</td>
<td>0%</td>
</tr>
<tr>
<td>14</td>
<td>Comb sharing</td>
<td>79%</td>
</tr>
<tr>
<td>15</td>
<td>Razor sharing</td>
<td>8%</td>
</tr>
<tr>
<td>16</td>
<td>I/V drug addiction</td>
<td>0%</td>
</tr>
<tr>
<td>17</td>
<td>Tattoeding</td>
<td>0%</td>
</tr>
<tr>
<td>18</td>
<td>Nose/Ear piercing from market</td>
<td>0%</td>
</tr>
<tr>
<td>19</td>
<td>HCV positive (diabetic)</td>
<td>19%</td>
</tr>
<tr>
<td>20</td>
<td>HCV positive male (diabetic)</td>
<td>26%</td>
</tr>
<tr>
<td>21</td>
<td>HCV positive female (diabetic)</td>
<td>74%</td>
</tr>
<tr>
<td>22</td>
<td>HCV positive (control group)</td>
<td>3%</td>
</tr>
</tbody>
</table>
Prevalence of hepatitis C in diabetic patients: a prospective study

Test methodology
After collection, blood samples (3 mL) were labeled and transferred to the laboratory for analysis. Each sample was centrifuged at 4000 rpm for 3-5 min. After centrifugation serum was separated from each specimen. These sera were tested immediately in 10 µL of negative control 3 wells, positive control 2 wells and remaining wells for specimens (total 96 microwells). For remaining 9 samples another the same kit was used. Hundred microliters of sample diluent was added to each well through micropipette (Analysis Technologies, USA). Microplate was covered with adhesive sheet and mixed well on vibrating mixer (Vortex Genie, USA). Then, wells were incubated at 37°C for 30 min. Then, the plate was taken out and washed with 350 µL of diluted washing solution for 5 times (10 s soak time for each wash) and all liquid was aspirated from each well. Subsequently, 100 µL of enzyme conjugate was pipetted in each well. Microwells plate was covered with adhesive sheet and incubated for 30 min at 37°C. Washing was repeated 5 times with 350 µL of diluted washing solution (10 s soak time for each wash) and all liquid was aspirated from each well and gently mixed the TMB substrate A and B in the ratio of 1 : 1 and 100 µL of the mixed substrate solution was added to each well. Wells were incubated for 10 min at room temperature. Hundred microliters of stop solution was then added to each well and absorbance was measured in the microplate reader (ELISA Reader Dia 710 Diamate, UK) at 450 nm with reference wavelength at 620 nm. A cut off value > 1 was considered reactive (7).

Statistics
Data analysis was carried out and descriptive analysis was performed. Results were expressed as percentages and mean. The binary logistic regression model was applied, which has given odds ratio (OR), 95% confidence interval (CI) and p-value (> 0.05 was considered significant statistically). Goodness-of-fit test was applied to determine that the applied regression model is the best fit.

Table 2. Distribution of HCV pattern in diabetic patients.

<table>
<thead>
<tr>
<th>No. of observation</th>
<th>Variables</th>
<th>Anti HCV antibody (positive)</th>
<th>Anti HCV antibody (negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Type of diabetes</td>
<td>16%</td>
<td>84%</td>
</tr>
<tr>
<td></td>
<td>Type 1</td>
<td>15%</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>Type 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Age group (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>≤ 35</td>
<td>16%</td>
<td>17%</td>
</tr>
<tr>
<td></td>
<td>36-45</td>
<td>32%</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>46-55</td>
<td>47%</td>
<td>41%</td>
</tr>
<tr>
<td></td>
<td>&gt; 55</td>
<td>5%</td>
<td>17%</td>
</tr>
<tr>
<td>3</td>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>26%</td>
<td>37%</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>74%</td>
<td>63%</td>
</tr>
<tr>
<td>4</td>
<td>Marital status</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Married</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>Unmarried</td>
<td>5%</td>
<td>5%</td>
</tr>
<tr>
<td>5</td>
<td>Locality</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Urban</td>
<td>89%</td>
<td>91%</td>
</tr>
<tr>
<td></td>
<td>Rural</td>
<td>11%</td>
<td>9%</td>
</tr>
<tr>
<td>6</td>
<td>Duration of diabetes (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1-5</td>
<td>21%</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>6-10</td>
<td>32%</td>
<td>28%</td>
</tr>
<tr>
<td></td>
<td>≥11</td>
<td>47%</td>
<td>27%</td>
</tr>
<tr>
<td>7</td>
<td>Blood transfusion</td>
<td>16%</td>
<td>17%</td>
</tr>
<tr>
<td>8</td>
<td>Hospital admission</td>
<td>84%</td>
<td>62%</td>
</tr>
<tr>
<td>9</td>
<td>Major surgery</td>
<td>63%</td>
<td>45%</td>
</tr>
<tr>
<td>10</td>
<td>Family history of HCV</td>
<td>16%</td>
<td>22%</td>
</tr>
<tr>
<td>11</td>
<td>Comb sharing</td>
<td>79%</td>
<td>79%</td>
</tr>
<tr>
<td>12</td>
<td>Razor sharing</td>
<td>16%</td>
<td>6%</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

The study population comprised of 100 diabetic patients and 100 healthy subjects recruited from the blood bank. The mean age of diabetic patients was 45.66 ± 10.94 years and the mean duration of diabetes was 8.27 ± 5.24 years.

Higher prevalence of HCV infection was estimated in females (74%) than males (26%). Most of the HCV positive patients were married (95%) and from urban locality (89%). Prevalence of HCV infection increased 47% with the increase in the duration of diabetes as maximum numbers of positive cases were found in the group of patients having ≥11 years of duration of diabetes. In HCV positive cases 84% patients have history of hospital admission and 16% have a history of blood transfusion, 63% have history of major surgery and 16% have a family history of hepatitis C. About 79% HCV positive patients share comb with others and 16% male patients share razors.

Percentage of HCV positive patients in different age groups is given in Table 2 for age group 1 (≤35 yrs), age group 2 (36-45 yrs), age group 3 (46-55 yrs).

Table 3. Binary logistic regression (Y versus X₁, X₂, .....X₁₅).

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE coefficient</th>
<th>Z</th>
<th>P</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>12.9918</td>
<td>3.33080</td>
<td>0.00</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>X₁</td>
<td>0.098731</td>
<td>1.06399</td>
<td>0.09</td>
<td>0.926</td>
<td>1.10</td>
</tr>
<tr>
<td>X₂</td>
<td>0.899143</td>
<td>0.900294</td>
<td>1.00</td>
<td>0.318</td>
<td>2.46</td>
</tr>
<tr>
<td>X₃</td>
<td>0.016980</td>
<td>0.0390291</td>
<td>0.44</td>
<td>0.664</td>
<td>1.02</td>
</tr>
<tr>
<td>X₄</td>
<td>1.48134</td>
<td>1.73071</td>
<td>0.86</td>
<td>0.392</td>
<td>4.40</td>
</tr>
<tr>
<td>X₅</td>
<td>0.244939</td>
<td>1.20350</td>
<td>0.20</td>
<td>0.839</td>
<td>1.28</td>
</tr>
<tr>
<td>X₆</td>
<td>-20.6236</td>
<td>33308.0</td>
<td>-0.01</td>
<td>1.000</td>
<td>0.00</td>
</tr>
<tr>
<td>X₇</td>
<td>-0.571161</td>
<td>0.0587632</td>
<td>0.74</td>
<td>0.457</td>
<td>1.04</td>
</tr>
<tr>
<td>X₈</td>
<td>-0.043702</td>
<td>0.799720</td>
<td>-0.71</td>
<td>0.475</td>
<td>0.56</td>
</tr>
<tr>
<td>X₉</td>
<td>1.69913</td>
<td>1.01311</td>
<td>1.68</td>
<td>0.094</td>
<td>5.47</td>
</tr>
<tr>
<td>X₁₀</td>
<td>0.189616</td>
<td>0.769522</td>
<td>0.25</td>
<td>0.805</td>
<td>1.21</td>
</tr>
<tr>
<td>X₁₁</td>
<td>-0.635654</td>
<td>0.861820</td>
<td>-0.74</td>
<td>0.461</td>
<td>0.53</td>
</tr>
<tr>
<td>X₁₂</td>
<td>2.79698</td>
<td>1.34305</td>
<td>2.08</td>
<td>0.037</td>
<td>16.39</td>
</tr>
<tr>
<td>X₁₃</td>
<td>2.79698</td>
<td>1.34305</td>
<td>2.08</td>
<td>0.037</td>
<td>16.39</td>
</tr>
<tr>
<td>X₁₄</td>
<td>2.79698</td>
<td>1.34305</td>
<td>2.08</td>
<td>0.037</td>
<td>16.39</td>
</tr>
<tr>
<td>X₁₅</td>
<td>2.79698</td>
<td>1.34305</td>
<td>2.08</td>
<td>0.037</td>
<td>16.39</td>
</tr>
</tbody>
</table>

Note 1: Y = Hepatitis C. 1. Coefficient of X₁ (type of diabetes) is 0.098, it means the prevalence of hepatitis C increases by 0.098 from type 1 diabetes to type 2 diabetes. 2. Coefficient of X₂ (gender) is 0.899, which means the prevalence of HCV is 0.899 more in females than males. 3. Coefficient of X₃ (age) is 0.016, which means the prevalence of hepatitis C in diabetics is increased by 0.016 with every unit increase in age. 4. Coefficient of X₄ (marital status) is 1.48, which means the prevalence of hepatitis C is 1.48 greater in married than unmarried. 5. Coefficient of X₅ (residential status) is 0.244, which means the prevalence of hepatitis C is increased by 0.244 from rural areas to urban areas. 6. Coefficient of X₆ (socioeconomic status) is 0.043, which means the prevalence of hepatitis C is decreased by 0.043 by unit increases (year) in duration of disease. 7. Coefficient of X₇ (duration of disease) is 0.043, which means the prevalence of hepatitis C is increased by 0.043 by unit increases (year) in duration of disease. 8. Coefficient of X₈ (blood transfusion) is -0.57, which means the prevalence of hepatitis C is decreased by 0.57 in patients having no blood transfusion. 9. Coefficient of X₉ (hospital admission) is 1.699, which means the prevalence of hepatitis C is increased by 1.699 by increasing hospital admissions. 10. Coefficient of X₁₀ (major surgical procedure) is 0.189, which means the prevalence of hepatitis C is increased by 0.189 in patients having major surgical procedure during their lives. 11. Coefficient of X₁₁ (family history of HCV) is -0.635, which means the prevalence of hepatitis C decreased by 0.0635 in patients having no family history of hepatitis C compared to patients having a family history of hepatitis C. 12. Coefficient of X₁₂ (razor sharing) is 2.79, which means the prevalence of hepatitis C is increased by 2.79 by razor sharing in male diabetic patients.

Note 2: In column of Odds ratios, the following items have been interpreted: 1. X₁ = type of diabetes OR = 1.10, which means type 2 diabetic patients have 10% more risk to develop hepatitis C than type 1 diabetic patients. 2. X₂ = gender OR = 2.46, which means female diabetic patients have 46% more risk to develop hepatitis C than male diabetic patients. 3. X₃ = age OR = 1.02, which means the risk of developing hepatitis C is increased by 2% with every unit (1 year) increase in age. 4. X₄ = marital status OR = 4.40, which means risk of developing hepatitis C is increased by 4% by every unit (1 year) increase in age. 5. X₅ = residential status OR = 1.21, which means risk of developing hepatitis C is increased by 21% in patients with history of major surgical procedures. 6. X₆ = socioeconomic status OR = 0.53, which means the risk of the development of hepatitis C is increased by 2% with every unit (1 year) increase in age. 7. X₇ = duration of disease OR = 1.04, which means risk of developing hepatitis C is increased by 4% by every unit (1 year) increase in age. 8. X₈ = blood transfusion OR = 0.56, which means the risk of the development of hepatitis C is decreased by 4% by every unit (1 year) increase in age. 9. X₉ = hospital admission OR = 5.47, which means risk of the development of hepatitis C is increased by 5.47 times with hospital admissions. 10. X₁₀ = major surgical procedure OR = 1.21, which means risk of developing hepatitis C is increased by 21% in patients with history of major surgical procedures. 11. X₁₁ = family history of HCV OR = 0.53, which means the risk of the development of hepatitis C is increased by 2% with every unit (1 year) increase in age. 12. X₁₂ = razor sharing OR = 2.79, which means the risk of the development of hepatitis C is increased by 2.79 by razor sharing in male diabetic patients.
Prevalence of hepatitis C in diabetic patients: a prospective study

The prevalence of hepatitis C in diabetic patients was higher as compared to control group of healthy blood donors (19% vs. 3%). The results of this study are in agreement with the results of some studies conducted earlier in Pakistan as well as in other countries (6-9). This higher prevalence of hepatitis C in diabetic patients may be the complication of diabetes therapy (10).

Furthermore, the prevalence of hepatitis C infection is higher in type 2 than type 1 diabetic patient (84% vs. 16%). Thus, the study establishes type 2 diabetes mellitus (D.M.) as a risk factor for hepatitis C, this study is in agreement with another study conducted earlier in Pakistan (7). Sixteen percent prevalence of HCV infection in type 1 diabetic patients reveals that there could be an association of type 1 D.M. upon the development of hepatitis C infection (8). It may be due to insulin therapy, which may increase the risk of acquiring viral hepatitis due to exposure of the needle (9).

It was observed that older patients have higher incidence of HCV infection as compared to those in younger age groups (9). This high seropositivity in the older age group may be due to more parenteral exposures and more chances of transmission of infection as compared to younger age group.

It was observed in this study that the risk of developing hepatitis C in diabetic patients increased with the increase in the duration of disease (4). This may be due to complication of diabetes therapy (2). The study also reveals that females have higher seropositivity as compared to males (74% vs. 26%). This result is in agreement with another study conducted earlier in Nigeria (9).

It was also evident from this study that certain factors as age, gender, marital status, residential status, history of hospital admission, blood transfusion and major surgery, duration of diabetes, comb sharing, razor sharing are important factors for the development of hepatitis C in diabetic patients.

CONCLUSION

According to the findings of this study it is concluded that there is a strong association between diabetes mellitus and hepatitis C but it is necessary to evaluate further whether D.M. is a risk factor for hepatitis C or vice versa. It is also clear from this study that certain factors, including female gender, older age, marriage, urban locality, history of blood transfusion, longer duration of diabetes, hospitalization, major surgical procedure, comb sharing, razor sharing increase the risk of developing HCV infection. Anti-HCV antibody and liver function tests should be done immediately after the diagnosis of diabetes and should be repeated periodically during anti-diabetic drug therapy. Special care and attention is required for parenteral therapy of diabetic patients to minimize the risk of HCV infection. It is suggested that this study can be further expanded to monitor liver function tests during anti-diabetic drug therapy to elucidate the development of liver disease which could be due to drug therapy or due to increasing duration of diabetes. Fourth generation ELISA test or PCR test should be done to detect HCV RNA in diabetic patients, which are more sensitive and reliable than 3rd generation ELISA test for the detection of anti-HCV antibodies.

REFERENCES


Received: 12. 05. 2015
Benign prostate hyperplasia (BPH) is one of the most common urological disorders diagnosed in aging male population. BPH is an overgrowth of both epithelial and smooth muscle cells within the prostatic transition zone and perirethral area (1, 2). That results in hypertrophic prostate tissue compression of the proximal urethra and in progressive increase of the lower urinary tract pressure, finally leading to an impaired urinary flow. The incidence of BPH is closely associated with age – it is a rare condition in men under the age of 40, but nearly 50% of 50+ men develop BPH symptoms, and it is...
diagnosed in nearly 80% of elderly patients (age 80 and over) (3, 4). This pathological condition results in – so called – lower urinary tract symptoms (LUTS), that may be divided into both obstructive (weak urine stream, straining, prolonged and incomplete voiding) and irritative (increased urinary frequency, urgency, urge incontinence, nocturia) ones (1, 5). Thus, BPH is regarded to be a clinical condition characterized by secondary bladder overactivity, similarly to primary, idiopathic overactive bladder syndrome (OAB), but induced by organic, subvesical obstruction and bladder hydrostatic pressure overload (1, 6).

Despite its high prevalence, the pathogenesis of BPH is only partially known. The current pathophysiological BPH description is focused on the hormonal deregulation with elevated dihydrotestosterone prostatic amount and enhanced secretion of the fibroblast growth factor (FGF) impairing processes of apoptosis. Those disturbances promote overgrowth of the prostate tissue. Much attention is also drawn to the prostatic production of highly tissue-damaging free radicals in response to local hypoxia (7). However, the chronic prostatic inflammation seems to be the key factor in BPH development. The evidence of prostatitis was found in approximately 90% of histological examination of samples collected during the transurethral resection of the prostate (8, 9). Moreover, the degree of inflammation was found to be correlated with the prostate volume and weight in patients with BPH (10). The origin of chronic prostate inflammation is ambiguous and some factors, such as: bacterial infections, viruses, sexually transmitted organisms, dietary factors, hormones, autoimmune response and urinary reflux are considered to both initiate and maintain the inflammatory response (8). That process disrupts the homeostasis between cellular death and proliferation, with final predominance of hyperplasia and reduction of the apoptotic process (1, 2). During BPH, an inflammatory reaction is also present in the urinary bladder, being co-responsible for irritative LUTS. A special role of arachidonic acid derivatives – prostaglandins and leukotrienes is currently emphasized in pathogenesis of inflammation, among various inflammatory mediators. Both isoforms of cyclooxygenases (COX) 1 and 2 (constitutive and inflammatory-inducible one, respectively) have been found in the prostate gland (11) and there are evidences that COX-2 mediated PG cause the increased expression of anti-apoptotic protein Bel-2 (12).

Lipoxygenase (LOX) isoforms are also overexpressed in prostate tissues, and they have been found in prostate cancer cells (13). Independently, most of inflammatory cells (e.g., polymorphonuclear leukocytes or macrophages) have also an ability to synthesize leukotrienes that are regarded to be strong chemoattractant factors, also promoting adhesion to the inflamed and damaged tissue (2).

The urinary bladder contains also abundant eicosanoids, commonly regarded to be important paracrine factors involved in the bladder control (14). Summing up, both prostaglandins and leukotrienes, as synthesized lipid inflammatory mediators, seem to be an attractive pharmacological target, and anti-leukotriene agents appear to exert the ameliorating effect in BPH and other conditions associated with bladder overactivity. That hypothesis was confirmed by Altavilla et al. (2), who demonstrated that the use of flavocoxid in rats with testosterone-induced experimental BPH resulted in substantial reduction of the prostate weight and hypertrophy along with blunted production of prostaglandin E\textsubscript{2} and leukotriene B\textsubscript{4}, with apoptosis stimulation and inhibited growth factor expression. Since the flavocoxid is a dual inhibitor of both cyclooxygenase and lipoygenase, their results suggest that modulation of eicosanoids production may be a rational approach to reduce LUTS in the course of BPH. However, it is uncertain whether the influence of eicosanoids on the autonomic nervous system (ANS) function may be also considered as an additional pharmacodynamics feature of novel, eicosanoid-targeted agents.

The autonomic nervous system function may be indirectly estimated using the heart rate variability (HRV) method based on the measurement of successive R-R (also called NN; normal-normal) intervals variation in ECG recordings in terms of temporal and spectral (frequency) analysis (15). BPH is relatively easily reproduced in an animal model (by surgical formation of a partial bladder outlet obstruction; PBOO) (16).

Thus, the aim of our study was to estimate the ANS function using the heart rate variability (HRV) in an experimental BPH model of partial bladder outlet obstruction (PBOO) in rats treated with the leukotriene receptor antagonist – montelukast (MLKT). Furthermore, we have also evaluated whether the expected, modulating impact of MLKT on the autonomic activity may contribute to the beneficial, desired effect of this compound. Hence, we have attempted to determine if due to its modulatory effect on the ANS activity, in addition to the anti-inflammatory effect, MLKT may be considered a potential drug used for alleviation of bladder dysfunction.
EXPERIMENTAL

Ethical issues
The medical experiment described in this paper was approved by the appropriate local ethics committee responsible for carrying out experiments on animals in Kraków.

Animals used for the experiment and a general plan of the study
The experiment involved 20 eight-week-old albino Wistar rats with an average body weight of 194.4 ± 7.9 g, obtained from the central animal house of the Faculty of Pharmacy UJCM. Upon arrival to the local animal house of the Department of Pathophysiology, during the first week, the animals acclimated to new living conditions. During that period, animals were housed in standard conditions, five individuals per cage, with unlimited access to water and food (Labofeed, Kcytnia). The experiment commenced on the second week with measurement of daily water intake by placement of intact rats in single metabolic cages. The partial bladder outlet obstruction (PBOO) surgery was performed on the following day in all animals. One subject fell during the surgery and another one on the second day of the postoperative period. Hence, 18 rats were finally subjected to further studies. The post-surgical recovery period lasted two days. On the third day, the daily intake of water was re-measured, and animals were randomly allocated to two groups: control (group 1; n = 9) or test group of animals treated per os with the leukotriene receptor antagonist - montelukast (group 2; n = 9).

Beginning on the fourth day after the PBOO procedure, the animals in the group 2 received MLKT. All animals demonstrated normal activity with no signs of postoperative complications. The post-surgical recovery period lasted two days. On the third day, the daily intake of water was re-measured, and animals were randomly allocated to two groups: control (group 1; n = 9) or test group of animals treated per os with the leukotriene receptor antagonist - montelukast (group 2; n = 9).

On days 4 to 14 after the PBOO induction, both control and test animals were kept in individual cages, with limited access to water (in the volume corresponding to the individual, average daily water intake, previously determined with repeated measurements) and with unlimited access to food. Animals in the group 2 received montelukast (obtained from commercial preparation - montelukast, Sandoz, sachets with 500 mg granulate containing 4 mg of active substance) dissolved in the measured volume of water. The montelukast dose of 2 mg/rat/day was applied, taking into account the average body weight of 217.0 ± 11.5 g of animals in the group 2 at the 15th day of the experiment, corresponding to 9.2 mg/kg body weight/day. The dose applied by us was consistent with the MLKT amount reported by other investigators - 10 mg/kg p.o. (17, 18), and also corresponds to the dose administered intraperitoneally - 10-30 mg/kg i.p. (19, 20). On the penultimate day of the experiment, all animals were once again placed in single metabolic cages with maintained access to the previously calculated volume water, and MLKT in group 2, to finally assess final body weight and the daily urine excretion. On the 15th day of the experiment, ECG recordings with subsequent HRV analysis were carried out for all animals to obtain standard time and spectral parameters. The procedure was completed under general pentobarbital anesthesia. Finally, after the ECG recording, animals were sacrificed with a lethal dose of pentobarbital and cystectomy was performed to determine the bladder wet weight (BWW) and to obtain bladder tissue for the subsequent histopathological assessment.

The PBOO surgery
The procedure of partial ligation of the proximal urethra (partial bladder outlet obstruction) was performed under general pentobarbital anesthesia (morbital, 60 mg/kg body weight (b.w.); calculated for the pentobarbital sodium). After making an incision in the midline of the body (in the umbilical ligament plan), an urinary bladder was unveiled, and following catheterization (epidural catheter, 1 mm diameter) the proximal section of the urethra was ligated (Dermalon 4-0). Then, catheter was removed leaving the urethra partially occluded and integuments were sutured (Dermalon 4-0). During first two post-surgical days, neomycin and oxytetracycline sprays were applied once a day to the postoperative wound.

HRV recordings
Twenty minute-long ECG records under general, pentobarbital anesthesia (morbital at a dose of 60 mg/kg b.w. calculated for the pentobarbital sodium) were performed for all study animals. The ECG signal, as a starting point for the subsequent HRV analysis, was acquired using pediatric electrodes Ag/AgCl (EK-S30 Sorimex PSG) and the Polygram system ADInstruments hardware and software, after prior hair removal and application of both abrasion paste and covering the skin with a standard ECG gel. Based on the obtained ECG recordings, standard time parameters: mean, max. and min. NN intervals, standard deviation of all NN intervals (SDNN), root mean square of successive differences (rMSSD) – all in [ms], and the mean heart rate HR [1/min] as well as spectral (frequency) ones: total HRV spec-
trum power (TP), power of the very low frequency component (VLF), power of the low frequency component (LF) and power of the high frequency component (HF) – all in [ms²] - were calculated (15, 21–25). We performed also the HRV spectrum normalization. It is a re-creation of the initially obtained spectrum to the secondary one with the VLF bypassing. Although the VLF power is a primary contributor to the total power (TP) development, by indicating the activity of slow thermoregulatory mechanisms, chemoreceptor stimulations or various neuroendocrine mechanisms (e.g., the renin-angiotensin-aldosterone system), it also includes some unknown, not fully understood, autonomic mechanisms. Therefore, the primary obtained HRV spectrum is usually subjected to re-calculation for share of relevant components (LF or HF) in total HRV power, excluding the VLF component power. That procedure allows determination of two normalized nLF and nHF parameters - expressed in normalized units ([n.u.]) (15, 21–25).

The spectrum bands for respective components was set as: 0.18 < VLF < 0.28 < LF < 0.78 < HF < 3, consistently with values applied by other researchers: Aubert et al. (26) (0.19 < LF < 0.74 < HF < 2.5), and Goncalves et al. (27) (0.10 < LF < 1.0 < HF < 3.0). Results were presented as the mean values ± SD.

Cystectomy and histopathological evaluation

In all study rats, the bladders were collected once the HRV recordings had been taken and following administration of a lethal sodium pentobarbital dose (160 mg/rat; calculated for pentobarbital sodium), to ascertain the changes in bladder wet weight (BWW) and its percentage weight in relation to the final body weight. Bladders were cut off with the proximal, ligated urethra. They were gently drained off and immediately weighted. According to the literature, cystitis may be evaluated not only by determination of changes in macroscopic and microscopic analysis but also by evaluation of the bladder wet weight. Thus, BWW is regarded to be an indirect marker of cystitis and bladder dysfunction (28, 29). When the BWW value was determined, collected bladders were placed in 4% formalin + PBS solutions for further histopathological assessment. The finally prepared microscopic sections were H + S stained to enable the histological evaluation of intensification of the inflammatory process.

Statistical analysis

Due to absence of normal distribution of obtained HRV parameters, statistical analysis was performed after their expression as natural logarithms. Parametric Student’s t-test with α = 0.05 was applied to evaluate the statistical significance. The conclusions of HRV results and statistical inference are given in Table 2 and Figure 1.

RESULT

The final body weight of study animals

In both study groups a trend of the body weight increase was observed throughout the experiment (15 days), compared to the value measured in the

| Table 1. The characteristic of studied animals with PBOO model. |
|-----------------------------------------|----------------|----------------|------------|
|                                       | Group 1        | Group 2        |            |
|                                       | control PBOO rats | PBOO rats + MLKT | Statistics |
| Starting body weight [g]               | 198.4 ± 7.9    |                |            |
| Final body weight on 14th day [g]      | 203.2 ± 8.6    | 217.0 ± 11.5   | NS         |
| Average daily water intake [mL]        | 20.7 ± 3.5     | 24.4 ± 6.3     | NS         |
| Bladder wet weight [mg]                | 145.0 ± 19.1   | 150.6 ± 40.9   | NS         |
| Bladder wet weight related to final body weight [%] | 0.058 ± 0.033 | 0.069 ± 0.017 | NS         |
| 24-h urine excretion on 14th day [mL]  | 6.9 ± 1.0      | 5.3 ± 1.5      | p = 0.02   |

PBOO – partial bladder outlet obstruction, MLKT – montelukast, NS – non significant.
beginning of the study. The final body weight in both control and MLKT-treated animals achieved similar values with no significant difference. Detailed results are presented in Table 1.

Average daily water intake and final 24-hour urine collection

The evaluated average daily water intake maintained during the following days of the experiment.
was similar in both studied groups. Thus, on days 4 to 14 each rat was supplied with an individually determined water volume with (group 2) or without (group 1) the addition of MLKT. Finally, on the day 15 a 24-h daily urine collection procedure indicated a lower daily urine volume in MLKT-treated animals compared to the value obtained in control rats. The difference was statistically significant. The results are presented in Table 1.

The bladder wet weight and histological assessment of collected bladders

Neither the bladder wet weight nor the calculated percentage of the bladder wet weight related to the final body weight differed significantly in study animals.

However, BWW value determined in parallel in intact animals (5 individuals) during our experiment was 120.1 ± 6.3 mg. That value significantly differed from the BWW results demonstrated in both control PBOO animals (145.0 ± 19.1 mg; p ≤ 0.05), as well as in PBOO animals treated with montelukast (150.6 ± 40.9 mg; p ≤ 0.05).

The higher BWW observed in PBOO rats can be considered as an indirect proof supporting the thesis of bladder tissue remodeling resulting from bladder adaptation to increased resistance due to the bladder outlet obstruction. Therefore, those results also confirm the efficacy of the performed PBOO surgery.

The detailed results relating to both study groups are presented in Table 1.

The time-domain HRV analysis

All time-domain HRV parameters, with exception of the range of NN intervals, were comparable in both groups and did not significantly differ between groups. Animals treated with MLKT demonstrated a globally increasing trend of both SDNN and rMSSD, although with no evidence of statistical significance.

The results of the time-domain HRV parameters with statistical inference are presented in Table 2.

The spectral HRV analysis

The performed spectral (frequency) HRV analysis revealed some statistically significant intergroup differences related to the power of the primary, non-normalized spectral components in the range of low (LF) and high (HF) frequencies. Higher values were observed in MLKT-treated rats (group 2). The observed changes concerning LF and HF components were not accompanied by the parallel change in the total power (TP) HRV spectrum. In addition, the non-normalized very low frequency (VLF) component power also did not signficantly differ between both analyzed groups.

Results of the spectral HRV analysis and values of non-normalized HRV components with statistical inference are presented in Table 2.

Another differences between studied groups were in values of nLF and nHF, which were statistically significantly higher and lower, respectively, in montelukast-treated rats compared to control animals. Those differences are shown in the Figure 1.

Histological analysis

A diffuse, minor and chronic inflammation was observed in stroma of the bladder mucosa in specimens of bladders collected from rats with PBOO receiving no montelukast. Focal signs of squamous epithelial metaplasia were found in some cases.

In all assessed specimens obtained from animals treated with montelukast (the PBOO + MLKT group) the histological presentation was normal.

DISCUSSION

The main findings of our experiment may be summarized as follows:

1. Rats with the experimental partial bladder outlet obstruction, treated with montelukast, were characterized by different autonomic nervous system activity compared to untreated animals within the same model. The 10-day long treatment with montelukast (leukotriene receptor antagonist) resulted in increased power of non-normalized LF and HF components with the total HRV power remaining unchanged. Moreover, the additional evidence of functional ANS rearrangement in MLKT-treated individuals was also evidenced by statistically significant nLF increase and nHF decrease in those animals, compared to control.

2. Rats with the experimental partial bladder outlet obstruction, treated with montelukast, after the 10-day long treatment excreted less urine daily compared to untreated animals and the difference was statistically significant.

3. In histopathological assessment, control group demonstrated some minor inflammatory changes, and MLKT-treated animals presented regular histological structure.

In accordance with generally accepted HRV guidelines (15), the power of respective spectral components as well as changes in time-domain HRV parameters reflect the activity of sympathetic and/or parasympathetic part of the ANS.

SDNN and TP correlate with the global autonomic tension whereas the range of NN variation,
rMSSD and HF are regarded to be markers of selected parasympathetic activity. LF is a spectral component frequency of which reflects various phenomena mediated by both sympathetic and parasympathetic parts of the ANS (15, 21–25).

Therefore, taking into account the observed HF increase in HRV spectrum of MLKT-treated animals, the hypothesis of parasympathetic overactivity in the conditions of antileukotriene blockade may be considered. However, it is ambiguous considering the simultaneous increase of the LF component in that group of animals. The observation may be also interpreted as a result of augmented sympathetic activity. Furthermore, analyzing the main time-domain HRV parameter associated with the parasympathetic tension (rMSSD), no significant change was observed. It is therefore obvious that the reasoning concerning the assessment of autonomic nervous system activity based on the results of both time and non-normalized spectral HRV analysis is uncertain. Hence, the next step of our analysis was based on the assessment of normalized HRV spectrum parameters, thought to reflect the selective parasympathetic (nHF) and sympathetic (nLF) tension.

Taking these premises into account, and in connection with equivocal results of the basic spectral analysis, we concluded that clear and statistically significant sympathetic predominance (nLF increase) along with parasympathetic withdrawal (nHF decrease) was observed in MLKT-treated animals. It could be then expected that experimental PBOO in preserving leukotriene action might be related to the reverse ANS functioning and leukotrienes would be considered as the agents contributing to a high parasympathetic and low sympathetic tension.

Moreover, the leukotriene action blockade, resulting in the abovementioned rearrangement of functional ANS status, with the observed sympathetic overdrive, could contribute to alleviation of bladder overactivity symptoms, that was consistent with observed changes in our daily diuresis measurements following a 10-day long MLKT administration.

Our results support also the overall concept concerning the role of arachidonic acid derivatives in the bladder pathophysiology. There is evidence that bladder overactivity results from abnormal bladder paracrine activity, associated with release of prostanoids of urothelial origin. Those agents are thought to influence the bladder function, both by their inflammatory action directed to the detrusor, and a secondary involvement in neuronal control of the bladder (30, 31). In general, according to the basic bladder neurophysiology, parasympathetic fibers and acetylcholine released from efferent terminals and affecting muscarinic receptors, are involved in bladder contraction and emptying, and their overstimulation may result in detrusor instability. On the contrary, sympathetic innervation and noradrenaline-mediated stimulation of adrenergic, β-3 bladder receptors contribute to detrusor relaxation and increase bladder compliance (30, 31). Moreover, a link between PGs and the muscarinic system has been described previously (32). Ikeda et al. (33) demonstrated also the inflammatory-facilitated afferent nerve activity via EP1 receptors in animal models. To sum up, stimulation of EP1 and EP3 receptors leads to contraction of urinary bladder smooth muscle cells, whereas stimulation of EP2 and EP4 receptors causes muscle relaxation (34).

Thus, arachidonic acid derivatives affecting autonomic fibers are important agents, co-responsible for bladder function control. In consequence, pharmacological agents abolishing prostanoid-induced bladder overactivity seem to be a future, potential therapeutic option in patients presenting LUTS. Among those agents, non-steroidal anti-inflammatory drugs (NSAIDs) are thought to be a new, promising therapeutic option, consistent with clinical observations of many researchers (35–38). NSAIDs seem to be a possible pharmacological choice in treatment of BPH patients due to their expected reduction in the severity of irritative LUTS, and to a lesser extent, of obstructive LUTS (39). Modulation of the EP1 and EP2 receptor activity (directly via EP1/EP2 antagonists or indirectly via COX inhibitors) could be also a therapeutic goal in urinary bladder dysfunction.

Moreover, NSAIDs are also consider to alleviate symptoms of bladder overactivity by autonomic activity modulation. In one of our previously published works (40) we evaluated the effect of piroxicam-induced (both in the low – 2 mg/kg b.w. and high – 10 mg/kg b.w. doses) prostaglandins synthesis block on activity of the ANS in bladder outlet obstruction. We revealed that piroxicam (PRX) in both doses produced a trend for reduction of value of all non-normalized components of HRV. The lower PRX dose caused an increased nHF value, and PRX administered in the dose of 10 mg/kg b.w. caused an increase of the nLF value.

In our other experiments we applied preferential COX-2 inhibitor - meloxcam (MLX; 5 mg/kg b.w.) to determine the effect of that compound on the ANS activity, in both PBOO (41) and CP-HC (42) models. We did not demonstrate any significant dif-
ferences in time and spectral HRV analysis in animals treated with MLX compared to the corresponding control groups. Therefore, these results suggest that COX-1 derived constitutive prostaglandins seem to exert neuromodulating properties and inhibition of prostaglandins synthesis with PRX administered at the dose of 10 mg/kg b.w. lead to functional reconstruction of ANS, with marked sympathetic predominance. That may contribute to reduction of the bladder contractile action and improvement of its compliance in the filling period, which was demonstrated by other authors in urodynamic tests for NSAIDs (40). Our findings are also further rationale for using NSAIDs in the treatment of bladder overactivity symptoms. However, the broad usage of NSAIDs in OAB-related disturbances treatment are still limited due to their serious side-effects when administrated systemically (p.o.) (39).

On a margin, our choice of PRX and MLX, belonging to NSAID subgroup constituting “oxi-cams” family, applied in our previous studies, resulted from the need for the using of active compounds with a slight difference in their detailed pharmacodynamics aspects, (considering their selectivity in relation to COX: non-specific vs. preferential, respectively) and preserving their maximal therapeutical and chemical structure similarity (expressing by the same main ATC code: M 01 A C).

We are aware of limitations of our experiment – our reasoning concerning the ANS function was based only on the indirect method of HRV and it has not been supported by any direct evidence, not even the plasma noradrenaline level measurements (as an indicative, laboratory marker of sympathetic activity).

In addition, conclusions regarding the role of leukotrienes in the pathogenesis of CP-induced cystitis were also made indirectly, based on the results obtained in terms of their receptor-blocking action. However, despite these limitations, our preliminary results suggest the necessity of further research, that should clarify the pathophysiological role of leukotrienes in BPH and other LUTS-related disturbances, as well as the therapeutical value of leukotriene-targeting antagonistic agents.

CONCLUSIONS

In the present paper we suggest that, in addition to prostaglandins, leukotrienes also play a modulatory role in autonomic control of the bladder, and the leukotriene antagonists also seem to be an effective therapeutical options in LUTS-associated disturbances.

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Conflict of interest

The authors declare that there is no conflict of interest in the authorship.

REFERENCES


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EVALUATION OF DRUG USE INDICATORS FOR NON-COMMUNICABLE DISEASES IN PAKISTAN

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Abstract: Irrational drug use practices are a burden to healthcare facilities. Poor prescribing practices affect the overall management and cost of treatment of non-communicable diseases that are the major cause of mortality and morbidity worldwide. In an effort to improve prescribing practices, this study was designed to assess prescribing, consultation and facility indicators in healthcare facilities of Punjab and Sindh provinces of Pakistan from December 2012 to December 2013. In this cross-sectional study, random and convenient sampling were used to collect data from both private and public healthcare facilities. Quantitative data were collected using structured questionnaire, observations and prescription analysis, whereas qualitative information on factors influencing prescribing practices was obtained by interviewing medical practitioners. A total of 13693 prescriptions were obtained from 500 patient-prescriber encounters. Results show that history taking, physical examination and diagnoses were adequate while generic prescribing was four-fold less than drugs prescribed by brands. Average number of drugs prescribed was 4.63 with more prescribing tendency in private facilities. 45.07% prescription costs were less than Rs. 150. Sulfonylureas, statins and ACE inhibitors were highly prescribed drugs for diabetes, hyperlipidemia and hypertension. Prescribing practices were dominantly influenced by severity of disease (73% Punjab; 81% Sindh), patient age (75% Punjab; 68% Sindh) and availability of drugs (62% Punjab; 56% Sindh) whereby 91% practitioners in Sindh and 52% in Punjab rely on medical representatives as the source of drug information. Moreover, the pharmacy and therapeutic committees in all facilities were non-functional along with non-availability of essential drug list in 87% health facilities. Thus, there are considerable opportunities to improve the rational use of medicines in Pakistan including low prices for generics, physician education, prescribing guidelines and formularies.

Keywords: non-communicable diseases, generics, prescribing indicators, Pakistan

Abbreviations: NCDs - non-communicable diseases, PTC - pharmacy and therapeutic committee, Rs. - rupees

Non-communicable diseases (NCDs) are the most important cause of mortality and morbidity worldwide. According to WHO status report, in 2008, 80% people died in low and middle income countries due to NCDs (1). Currently, the research is focused to assess the implications of NCDs in low and middle economic countries (2). Pakistan, a middle income country is constantly fighting with multiple diseases including osteoporosis, hypertension, hyperlipidemias, peptic ulcer and diabetes. According to national health survey, every third person over the age of 40 is vulnerable to a wide range of diseases in Pakistan. Only 12.5% cases of hypertension are adequately managed while the rest become a burden to daily life of patients (3). The prevalence of osteoporosis, peptic ulcer and diabetes is very high in Pakistan (4-6). Obesity rises inpatient costs by 46%, and prescription drugs by 90%. The critical impact of obesity in the next decade will likely lead to staggering costs and a sig-

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nificant medical burden on health care system (7). A similar trend has been observed for osteoporosis with an increase in fracture prevalence among up to three times greater in young women (4).

In 2011, researchers made an effort to discuss the risk factors associated with NCDs at United Nations High Level Summit. There was limited information on distribution of disease burden in low and middle income countries. Moreover, prevention and management of diseases in low-resource settings are mired by a lack of consideration to socioeconomic situations (8-10). Therefore, we need to evaluate the health care quality that the patients are receiving for these diseases. This quality evaluation is receiving worldwide attention and has become pivotal to improve the health care of patients (11, 12). Assessing the prescribing, drug use and consultation patterns with the WHO indicators is necessary to promote rational drug use in developing countries (13).

WHO indicators allow the comparison of parameters among various health facilities and evaluate the population’s medication needs. This aids in highlighting irrational prescribing patterns that lead to ineffective treatment, exacerbation of illness, distress to the patient and higher costs. Such practices are perpetuated through patient demand, peer practices and salesmanship by medical representatives (14). The availability of essential drugs and pharmacy and therapeutic committee (PTC) can improve the health status and prescribing practices. There is an essential drug list in Pakistan which contains 335 medicines; however, access to those is limited due to ineffective quality assurance, poor affordability and irrational use. These are compelled by flaws in pharmaceutical regulation dealing with quality and cost effectiveness in drug registration, a lack of transparent pricing policy, unregulated provider prescriptions and weak supply management (15).

It has been estimated that drugs cost up to 60% of total healthcare expenditure (16). Patients have to pay a substantial hidden cost of medicines due to low drug availability at public sector facilities as they are often forced to purchase from private retail pharmacies. Mean spending per prescription was found to be Rs. 252 at private sector facilities compared to Rs. 198 at public sector facilities (15). Significant savings could be attained by swapping private sector purchases from originator brand medicines to lowest priced generic equivalents (16). Potential ways instigated by health authorities to address this include encouraging the prescribing of low-cost generics, especially where generics are priced at 2% to 10% of pre-patent loss prices such as the Netherlands, Sweden and the United Kingdom (17). Enhancing adherence to the medicines prescribed will improve prognosis, reduce long term costs and increase physician familiarity with the medicines they prescribe. This can be achieved through the use of formularies and prescribing guidelines thereby reducing costs associated with management of adverse drug reactions and drug interactions (18, 19).

In an effort to describe and quantify the situation, we have conducted this study to evaluate the prescribing practices of medical practitioners in Sindh and Punjab provinces of Pakistan. We have determined the current treatment practices, effect of patient parameters, cost analysis and factors influencing prescribing patterns especially for management of hypertension, hyperlipidemia, peptic ulcer, osteoporosis and diabetes.

**METHODOLOGY**

The present study was conducted in two districts: Sindh and Punjab, of Pakistan during the period from December 2012 to December 2013. In this cross-sectional study, convenient sampling was used to collect data from private and public health care facilities of 6 large cities in selected provinces. Cities were selected based on random sampling. Both qualitative and quantitative data were collected using a structured questionnaire developed according to the WHO guidelines (13). Information in questionnaire included demographic and socioeconomic data of patients, prescribing indicators, facility indicators, cost of prescriptions and sources of information of medicines. All questions were clearly defined and mutually exclusive. Consultation indicators were assessed by structured observations and qualitative data on factors influencing the prescribing practices was gathered by interviewing the medical practitioners. Coding, type, theme, numbering and sequence of answers were clearly designed so that response could be easily converted to variable and subjected to statistical analysis. This study was conducted with the consent of medical practitioners informing them that it is solely conducted for academic purposes without any intention of modifying their prescribing behavior. Patients were informed about the aims and process of the study, and written informed consent was obtained from all patients. The anonymity of volunteers was maintained. Furthermore, it was approved by the Ethics Committee of the Department of Pharmacy and Board of Studies of Sargodha University, Sargodha, Pakistan.
The questionnaire was divided into four sections. First section contained facility ID, patient ID and prescribing indicators including drug names (brand/generic), dosage, strength, duration and mode of administration, cost and presence on essential drug list. Second section was about patient care and disease management indicators including consultation time, dispensing time, number of drugs prescribed and dispensed, number of drugs adequately labelled, patient’s knowledge of dosing instructions, diagnosis, history, physical examination, laboratory test ordered and required, patient referrals and treatment in accordance with standard protocols. Third section recorded information on facility and practitioner in terms of facility name/ID, type (public/private), location (rural/urban), level (primary/secondary/tertiary), availability of essential drugs list, pharmacy and therapeutic committee (PTC), practitioner’s name, gender, qualifications, experience in years, sources of information on drugs and factors affecting prescribing practices. Fourth section was used to collect data from patients on patient care indicators and patient’s perspective regarding health facility/practitioner’s practice.

A total of 13693 prescriptions were collected from 500 medical practitioners. We emphasized on the management of osteoporosis, hypertension, diabetes, peptic ulcer and hyperlipidemias. Trained pharmacists and pharmacy students visited the health facilities until the required quota of prescriptions was obtained from the respected facility. A pilot study was conducted in another district to enhance the quality and robustness of the survey instruments.

The quality of the prescriptions was assessed based on history taking, physical examination, informing diagnosis to patients, lab tests and standard treatment protocols. A team of interviewers

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<td>Self-reported health</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>5132</td>
<td>3592</td>
<td>1540</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fair</td>
<td>4245</td>
<td>2674</td>
<td>1571</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Poor</td>
<td>4316</td>
<td>1330</td>
<td>2986</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 1. Demographic data of the patients.

Table 2. Socioeconomic parameters and health status of the patients.
especially trained for this purpose observed each patient-prescriber encounter to gather respective data. Medical practitioners were interviewed to assess factors modifying prescribing patterns categorized into different themes. The availability of WHO essential drug list, Pakistan essential drug list and PTC was also assessed in health facilities.

Data analysis was done using Statistical Package for the Social Sciences (SPSS-16) and presented as percentages. Paired t-test was used to compare the results between Sindh and Punjab provinces and were considered statistically significant at $p < 0.05$.

RESULTS

The present cross-sectional study conducted in Sindh and Punjab provinces of Pakistan demonstrates significant ($p < 0.05$) variation in prescribing, facility and consultation indicators between the two provinces. A majority of the patients were between 51-70 years of age (Table 1) having Rs. 11000 to 20000 income range (Table 2). In Punjab, most of the patients (3529 patients) reported themselves having good health while in Sindh a majority reported to have poor (2986 patients) health status (Table 2).

The average number of drugs prescribed were almost similar ($p > 0.05$) between the provinces. Other prescribing and consultation indicators except required lab tests and drugs prescribed by public sector practitioners were significantly ($p < 0.05$, $p < 0.001$) different between Sindh and Punjab health facilities (Table 3). Sulfonylureas remain the major drugs prescribed to antidiabetic patients in Sindh while metformin is the highest prescribing drug in Punjab. ACE inhibitors in Punjab and diuretics in Sindh are used for hypertension and stains for hyperlipidemias in both provinces (Table 4). The use of bisphosphonates and proton pump inhibitors are also significantly ($p < 0.05$, $p < 0.001$) different between the provinces (Table 4).

Table 5 contains data on the cost of drugs prescribed where 45.07% of prescriptions were less than Rs. 150 and least number of prescription were ranged between Rs. 351-450. The qualitative data

<table>
<thead>
<tr>
<th>Indicators</th>
<th>No. of prescriptions n = 13693</th>
<th>Punjab</th>
<th>Sindh</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adequate history obtained</td>
<td>7920 (57.84%)</td>
<td>4631</td>
<td>3289</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Relevant physical examination done</td>
<td>7584 (55.38%)</td>
<td>4974</td>
<td>2610</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Diagnosis informed to patient</td>
<td>11394 (83.21%)</td>
<td>7425</td>
<td>3969</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Diagnosis written on the prescription</td>
<td>11743 (85.76%)</td>
<td>7001</td>
<td>4742</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Lab tests ordered</td>
<td>5832 (42.59%)</td>
<td>2589</td>
<td>3243</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>Lab tests required</td>
<td>4301 (31.41%)</td>
<td>2053</td>
<td>2248</td>
<td>Ns</td>
</tr>
<tr>
<td>No. of drugs prescribed</td>
<td>63408</td>
<td>34789</td>
<td>28619</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>No. of drugs prescribed by brand names</td>
<td>49581 (78.19%)</td>
<td>27144</td>
<td>22437</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>No. of generics prescribed</td>
<td>13827 (21.81%)</td>
<td>7645</td>
<td>6182</td>
<td>$&lt; 0.05$</td>
</tr>
<tr>
<td>No. of drugs prescribed by public sector medical practitioners</td>
<td>29367</td>
<td>14926</td>
<td>14441</td>
<td>Ns</td>
</tr>
<tr>
<td>No. of drugs prescribed by private sector medical practitioners</td>
<td>34041</td>
<td>19863</td>
<td>14178</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>Average no. of drugs prescribed</td>
<td>4.63</td>
<td>4.65</td>
<td>4.61</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Ns - not significant
gathered by interviewing the medical practitioners showed that the most dominating factors modifying prescribing practices were severity of disease, patient age, availability of drugs at health facility and socioeconomic status of the patient. Moreover, excess stock of drugs at the hospital pharmacy stores was also a contributing factor in prescribing a particular brand (Table 6). Among various sources of information on medicines, medical representatives are the principal source on which practitioners rely to prescribe a drug. Scientific journals, newsletters, therapeutic indices and standard guidelines are less consulted by practitioners. 87% of health facilities neither have WHO essential drug list nor Pakistan essential drug list. PTC were established in 42% of health facilities but these were also non-functional.

**DISCUSSION**

Chronic diseases are major cause of mortality and morbidity globally. These can be controlled effectively if medicines are easily accessible and affordable. Yet, most of the population of low and middle income countries lack access to essential medicines. In line with these facts, we conducted a survey to evaluate the rationality of prescribing practices of medical practitioners in two large provinces of Pakistan. It was found that a majority of patients has access to medicines for the treatment of hypertension, hyperlipidemias, osteoporosis, peptic ulcer and diabetes and only 9.37% prescriptions were above Rs. 551. The preliminary screening of disease was adequate to diagnose correctly and effective medicines are used for the management of non-communicable diseases. Despite all this, it is a matter of concern that thousands of people in Pakistan are suffering from NCDs. Research conducted by World Economic Forum has estimated that over the period 2011-2025, low and middle income countries could lose 4% of average GDP per year due to morbidities caused by NCDs (20).

There was significant prescribing of bisphosphonates compared to proton pump inhibitors, antidiabetics, antihypertensive and antidyshlipidemics. This indicates a trend in prescribing of bisphosphonates along with vitamins and calcium supplements in patients prone to osteoporosis (21). Sulfonylureas and metformin are currently common medicine for the treatment of type 2 diabetes mellitus. A majority of the patients are prescribed these drugs despite availability of better and safer alternatives such as thiazolidinediones and exenatide. Studies have shown that the treatment with sulfonylureas, glinides, and pioglitazone cause body weight

<table>
<thead>
<tr>
<th>Therapeutic class of drugs</th>
<th>No. of drugs</th>
<th>Punjab</th>
<th>Sindh</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisphosphonates</td>
<td>10653 (16.80%)</td>
<td>7255</td>
<td>5417</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Proton pump inhibitors</td>
<td>7090 (11.18%)</td>
<td>3466</td>
<td>2916</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Antidiabetic</td>
<td>5271 (8.31%)</td>
<td>2896</td>
<td>2375</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Sulfonylurea</td>
<td>3928</td>
<td>1983</td>
<td>1945</td>
<td>Ns</td>
</tr>
<tr>
<td>Metformin</td>
<td>3736</td>
<td>2162</td>
<td>1577</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Thiazolidinediones</td>
<td>1745</td>
<td>931</td>
<td>815</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Acarbose</td>
<td>945</td>
<td>462</td>
<td>483</td>
<td>Ns</td>
</tr>
<tr>
<td>Exenatide</td>
<td>542</td>
<td>389</td>
<td>153</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Insulin</td>
<td>302</td>
<td>167</td>
<td>135</td>
<td>Ns</td>
</tr>
<tr>
<td>Antihypertensives</td>
<td>6232 (9.83%)</td>
<td>4081</td>
<td>2151</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>4625</td>
<td>2863</td>
<td>1762</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Beta-blockers</td>
<td>2185</td>
<td>1357</td>
<td>1878</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Diuretics</td>
<td>3996</td>
<td>1972</td>
<td>2024</td>
<td>Ns</td>
</tr>
<tr>
<td>Antihyperlipidemics</td>
<td>5542 (8.74%)</td>
<td>2501</td>
<td>3041</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Statins</td>
<td>4274</td>
<td>2591</td>
<td>1667</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Resins</td>
<td>1329</td>
<td>1058</td>
<td>271</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Fibrates</td>
<td>847</td>
<td>439</td>
<td>408</td>
<td>Ns</td>
</tr>
</tbody>
</table>

Ns - not significant
gain while sulfonylurea therapy is also associated with a risk of hypoglycemia. Metformin is first-line diabetes treatment; however, therapy amplification with either insulin or a sulfonylurea is considered an efficacious strategy with reasonable costs (22).

The use of exenatide is also escalating gradually in Pakistan for the management of patients with inadequate glycemic control by sulfonylureas and metformin. Though the number is far less in Punjab and Sindh, yet, its effectiveness has made its way to its prescribing. A study has shown that once weekly exenatide is more effective than insulin in achieving target weight loss and HbA1C along with low risk of hypoglycemia in uncontrolled diabetes (23). Furthermore, ACE inhibitors are taking a lead for the treatment of hypertension in Pakistan. It is common for diabetic patients to develop cardiac complications especially hypertension in the long run. ACE inhibitors are the first line choice in this scenario (24). Medical practitioners prefer ACE inhibitors over other antihypertensives as their first choice. However, diuretics are also prescribed as primary drug by practitioners in Sindh. Statins are commonly prescribed to modulate cardiovascular complications and risk associated with obesity in diabetics (25). In our study, we have observed that they are prescribed in patients with hypertension and diabetes with obesity. The prescribing of statins is a good indicator for its rational use for prevention and treatment of dyslipidemias. However, its limited prescribing in Pakistan is a concern as NCDs such as diabetes have much higher prevalence than the current use of stains. Along with this, the presence of proton pump inhibitors in 11.18% prescriptions indicates fair prevalence of drug induced or other forms of peptic ulcer in people of Sindh and Punjab. We have observed that medicines are being prescribed by brands rather than generics. This is driven by belief that generic medicines are less safe than brands, patients demand, availability of drugs at

Table 5. Cost analysis of drugs prescribed in health facilities of Punjab and Sindh.

<table>
<thead>
<tr>
<th>Cost (Rs.)</th>
<th>No. of prescriptions (%)</th>
<th>Punjab</th>
<th>Sindh</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 &lt;</td>
<td>6172 (45.07)</td>
<td>3307</td>
<td>2865</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>151-250</td>
<td>2734 (19.97)</td>
<td>1757</td>
<td>977</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>251-350</td>
<td>1934 (14.12)</td>
<td>1391</td>
<td>543</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>351-450</td>
<td>546 (3.99)</td>
<td>318</td>
<td>228</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>451-550</td>
<td>1024 (7.48)</td>
<td>512</td>
<td>522</td>
<td>Ns</td>
</tr>
<tr>
<td>Above 551</td>
<td>1283 (9.37)</td>
<td>785</td>
<td>498</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

Table 6. Factors modifying prescribing practices of medical practitioners (n = 500).

<table>
<thead>
<tr>
<th>Influencing factor</th>
<th>Response % of Punjab</th>
<th>Response % of Sindh</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Socioeconomic status of the patient</td>
<td>67%</td>
<td>41%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Previous experience of treating patients</td>
<td>37%</td>
<td>34%</td>
<td>Ns</td>
</tr>
<tr>
<td>Severity of disease</td>
<td>73%</td>
<td>81%</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Patient demand for specific drugs</td>
<td>34%</td>
<td>17%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Availability of drugs at facility</td>
<td>62%</td>
<td>56%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Placebo effect of drugs</td>
<td>6%</td>
<td>11%</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Age of the patient</td>
<td>75%</td>
<td>68%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Concern of losing patients to others practitioners</td>
<td>29%</td>
<td>51%</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Others</td>
<td>8%</td>
<td>13%</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

Ns - not significant
facility and low quality threshold for registration of medicines in Pakistan (15, 26). The severity of disease, socioeconomic factors, patient age and medicine availability were affecting the prescriber’s choice of medicine in Punjab. In Sindh, prescribers were also concerned over losing their patients to other practitioners if they did not prescribe a reputable brand of drug. These factors among others also contribute towards the prescriber’s choice of prescribing by brands over generic names. Another important factor especially in Sindh was that medical practitioners dominantly rely on medical representatives as a source of drug information. The low percentage of prescriptions by their generic name adds to high cost of drugs to patients especially as good quality generics, e.g., generic omeprazole and simvastatin, are priced as low as 2% to 4% of pre-patent loss prices in the Netherlands, Sweden and the UK (27, 28).

Another major concern was unavailability of essential drugs lists and non-functional PTCs in health facilities in Pakistan. PTCs are responsible for drug-use evaluation, adverse drug event monitoring and reporting, approval of guidelines for medication management and ensuring the safe, appropriate, and cost-effective use of pharmaceuticals (29). The establishment of functional PTCs, formularies and standard treatment guidelines should be seen as part of a long-term campaign in Pakistan to enhance the appropriate management of patients with chronic diseases. These activities should improve prescribing practices are influenced by the drugs on the formulary or essential drug lists and their availability at each facility (30).

Limitation of this study is that we did not correlate diagnosis, treatment and outcomes in our analysis. But, the health facilities in Pakistan does not run a comprehensive drug utilization system to manage patient drug profile and outcomes in any sector either public or private. This is the future perspective where community-based programs, greater focus on prevention within primary care together with monitoring, reporting and accountability mechanisms in healthcare system are critical elements in maintaining healthier lifestyle.

CONCLUSION

In conclusion, the present study describes the prescribing practices of medical practitioners in Sindh and Punjab provinces of Pakistan where prescribing by brand names, influence of medical representatives, unavailability of essential drugs lists, non-functional pharmacy and therapeutic committees, and availability of drugs at health facility are issues in healthcare system. All these contribute to higher cost of treatment. Despite that we also found that medical practitioners are prescribing standard treatments to manage major diseases in Pakistan, yet, we are far behind in controlling all NCDs.

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Conflict of interest

There are no conflicts of interest declared by authors.
REFERENCES


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THE USE OF PANAX GINSENG AND ITS ANALOGUES AMONG PHARMACY CUSTOMERS IN ESTONIA: A CROSS-SECTIONAL STUDY

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2Estonian Literary Museum, 42 Vanemuise St., 51003 Tartu, Estonia

Abstract: The aim of the cross-sectional study was to evaluate the pattern of complementary self-treatment with P. ginseng and its analogues amongst pharmacy customers in Estonia. The study instrument consisted of multiple-choice items related to personal knowledge about and experience with the use of P. ginseng and its analogues. In total, 1233 customers participated in the study. Of study participants, 18.1% reported the use of P. ginseng and its analogues in their lives. P. ginseng preparations were used mostly according to the well-known indications (tiredness, weakness and decreased mental and physical capacity). Of P. ginseng users 44.3% reported positive treatment effects and 12.0% had experienced different side effects. With increase of age (p < 0.01) and at lower levels of education (p = 0.04), the use of ginseng or its analogues decreased. The better the users evaluated their health, the better they perceived the effect of P. ginseng preparations (p < 0.01). This study reported rather frequent use of P. ginseng and its analogues. P. ginseng could be seen in the treatment of conditions, where the use of local medicinal plants has not been established. Further research is needed to learn more about public knowledge and experiences about efficacy and safety of P. ginseng and its analogues.

Keywords: Panax ginseng, ginseng analogues, self-treatment, pharmacy customers

The root of the Panax ginseng Meyer has been a popular and widely used traditional herbal medicine in Asian countries for thousands of years. The popularity of P. ginseng as natural Chinese medicine has increased globally and currently it is frequently available in Western herbal preparations (1). P. ginseng is reported to have a wide range of therapeutic applications – it has beneficial effects on cardiovascular system, central nervous system, and immune system (2). Nevertheless, “there is clearly a lack of definitive evidence demonstrating any effects of P. ginseng on human physical and mental performance or any parameter related to perceived energy” (3, 4), nor the evidence to claim all health benefits and insure safety issues, as studies are often contradictory (4), which in fact is the case with many complementary and alternative medicines (CAM’s) used worldwide (5). In addition to the contradictory clinical information about P. ginseng, only few international studies have focused on the user perception about P. ginseng (6).

P. ginseng arrived to Europe in the early Middle Ages through Arabian merchants. However, due to little awareness about medical properties of P. ginseng, it became known only since 17-th century. Nevertheless, medical doctors did not trust the miracle remedy and P. ginseng was not included to most European pharmacopoeias till 19-th century. A new wave of popularity of P. ginseng has grown during past fifty years.

According to the European Medicines Evaluation Agency (7), P. ginseng, as one-component preparation, has been available in different pharmaceutical formulations (tablets, powder, hard and soft capsules, oral liquid and herbal instant tea) in Germany, France and Sweden at least since middle of 1970s; in Austria, Ireland and Spain since 1980s and in Denmark, Poland and Portugal since 1990s. There is a large variability in the number of different P. ginseng preparations available on the market of different European countries starting with 60 different preparations in Germany and ending with one to two in Austria, Belgium, Denmark, Ireland and Portugal. In Italy and Latvia only combination preparations containing P. ginseng and/or vitamins, minerals and other components are available. According to regulatory status, P. ginseng preparations are granted marketing authorization as medicinal products in Austria, Belgium, Denmark, France, Germany, Ireland, Latvia, Poland, Portugal

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and Spain. *P. ginseng* preparations are considered as traditional preparations in Sweden and partly in Germany, Poland and Spain (7).

In Europe, *P. ginseng* is definitely one of the medicinal plants which have contributed to the adaptation of new exotic species into local CAM’s (8). Similarly to the description given in several international studies about the use of *P. ginseng* (9-12), this medicinal plant is considered well-known and frequently used tonic available in community pharmacies also in Estonia.

Estonia, a small country in the Northern Europe, is one of the three Baltic States and bordering with Finland, Russia and Latvia. About 70% of its 1.3 million population are Estonian-speaking, while the rest of the population mostly speaks Russian as their mother-tongue. Estonia is known for intensive historical and modern use of local native and cultivated medicinal plants for self-medication of different minor conditions (13-17). In addition, foreign medicinal plants (such as *Mentha piperita*, *Chamomilla recutita*, *Calendula officinalis* and *Salvia officinalis*) have been adopted to the local culture or substituted with wild medicinal plants (18). There are several alternative means of information retrieval about the use of medicinal plants: traditions or literary sources, previous personal experience as well as information provided by healthcare professionals, usually pharmacist or doctor (19-21).

Based on its history and location, Estonia has been crossing of eastern and western culture and traditions. This could also be seen in the history of the use of *P. ginseng* in Estonia. *P. ginseng* is a good example of a foreign plant with no historical use detected in earlier local folklore (22). *P. ginseng* as a wild species does not grow in Estonia and to date cultivation of this medicinal plant has not been established also (23). However, the *P. ginseng* preparations have been available at local community pharmacies starting from the last decades of 19-th century as the latest (24). At that time, Estonia was incorporated to the Russian Tsarist Empire. Russia, known as country with traditional guidelines for using *P. ginseng* (25), made these preparations also accessible in the western regions of its territory. During the First Republic of Estonia (1918–1940) the economic and cultural contacts were developed mostly with Western Europe and this could be one of the reasons, why *P. ginseng* was not available at community pharmacies, but was introduced again after the Second World War in the middle of 1950s, when Estonia was part of the Soviet Union (26). All *P. ginseng* preparations now available in Estonia are prepared from *P. ginseng*. While during Soviet period the promotion of medicinal products was unknown in Estonia, the commercials were available in mass-media after regaining of independence from the beginning of 1990s. In one of these commercials aged Chinese men demonstrated the human-like root of *P. ginseng* and introduced the ginseng preparations to the Estonian people.

There have been some analogues of *P. ginseng* such as *Eleutherococcus senticosus* (Rupr. & Maxim.), *Rhodiola rosea* L. (synonym *Sedum rosea* (L.) Scop.) and *Schisandra chinensis* (Turcz.) Baill., traditionally used during Soviet period (1940-1991) in Estonia (27) and/or *tinctures*, liquid extracts, tablets or capsules of described medicinal plants are available also in present time.

In some countries, including Estonia, ginseng has been perceived as a cultural representation of Chinese Medicine. Although, the medicine is well known and widely available, the public perception on the use of ginseng and its analogues are poorly studied in international literature.

To fill this knowledge gap, this study was aimed to evaluate the pattern of complementary self-treatment with *P. ginseng* and its analogues (*Eleutherococcus senticosus*, *Rhodiola rosea*, and *Schisandra chinensis*) amongst pharmacy customers in Estonia.

**METHODS**

**Study design**

Convenience sample of Estonian and Russian speaking pharmacy customers in three different towns of Estonia was used. Tartu (located in South-Estonia, population about 100,000) is the second biggest town in Estonia, where the majority of permanent residents are Estonian speaking. In the capital city of Estonia, Tallinn (located in North-Estonia, population about 400 000), the number of Russian speaking permanent residents is higher. In Kohtla-Järve (located in North-East Estonia close to Russian boarder, population about 40,000) a majority of the population are Russian speaking. In Tallinn, the study was undertaken in three community pharmacies in other two towns only one pharmacy was selected. All community pharmacies can be classified as medium size (approx. 30,000 prescriptions per year) pharmacies with large selection of non-prescription medicines and herbal products.

Two independent researchers conducted the study at the same period (June-August) in Tartu and Kohtla-Järve (2011) and in Tallinn (2012). In all community pharmacies customers approaching to the...
counter of non-prescription medicines were invited to participate in the study. The research assistant explained the purposes of the study and oral informed consent was received from all interviewed pharmacy customers. In both, Tartu and Kohtla-Järve community pharmacies, 300 respondents were interviewed, in Tallinn 650 respondents were involved in the study. In total, 1250 pharmacy customers participated in the study. However, as 17 questionnaires were incomplete, the analysis was carried out with 1233 replies. The number of pharmacy customers which refused to participate in the study was not documented. For those respondents who never used Panax ginseng or its analogues, only demographic data were collected. Respondents were interviewed in their native language (either Estonian or Russian) and only local residents participated in the study.

Study instrument

The study instrument was developed and discussed by a panel of researchers with a pharmaceutical background and extended knowledge in phytotherapy, ethnobotany, social pharmacy and semiotics. The study instrument applied was developed by using previous study models for evaluating self-medication with OTC medicines including medicinal plants (17, 20, 21, 28). A copy of the study instrument can be obtained from the corresponding author.

The study instrument consisted of multiple-choice items related to (1) personal knowledge or experience of the use of Panax ginseng, (2) time and duration of the use, (3) specific preparation or form used, (4) purpose of the use and perceived effects, (5) mode of action and side effects of Panax ginseng and its analogues, and (6) demographic characteristics of the respondent.

In Europe, Panax ginseng preparations are used as a tonic in case of tiredness, weakness and decreased mental and physical capacity (7). These indications have been presented in package information leaflets of Panax ginseng preparations marketed in Estonia and were pre-listed to assist respondents in selection of appropriate descriptions. In addition respondents could describe other indications, for what Panax ginseng was used.

In addition to Panax ginseng use, the study participants were asked to recall the use and efficacy of its analogues. Three different species were listed by their vernacular names in study instrument as to understand what analogues were used: E. senticosus, R. rosea and S. chinensis.

To assist the identification of the preparations nine different photos of preparations of Panax ginseng available at the period of study were demonstrated to the respondents (Fig. 1). The pictures worked as triggers for activating mental herbal of the respondents (term proposed by Kołodziejska-Degór ska) (29). Based on the experience of this study, several respondents recalled their use of Panax ginseng only after seeing the pictures of the preparations. Panax ginseng analogues were not illustrated with actual preparations.

Figure 1. Pictorial representations of the Panax ginseng preparations which were available and were shown to the respondents during the interview: 1. Gerimax tablets of ginseng extract; 2. Gerimax tablets of ginseng extract with vitamins and minerals; 3. Gerimax liquid extract of ginseng; 4. Ginseng N liquid extract of ginseng; 5. Dynamisan tablets of ginseng extract; 6. Strong GinSeng tablets of ginseng extract; 7. Zhenchenya nastoika tincture of ginseng; 8. Vishpha tincture of ginseng; 9. Spectrum capsules of ginseng with vitamins, minerals, rutin and lecithin
The content validity and comprehensibility of the study instrument was pre-tested by ten randomly selected pharmacy customers of participating community pharmacy in Tartu. Minor changes to the wording of the items were made, based on feedback received.

This research did not use identifiable human material or data. The survey conforms to the Declaration of Helsinki and the ethical standards of Estonia. The researchers have fulfilled the Data Protection Act of Estonia.

Statistical analysis

Initial data were coded, inserted and stored in MS Excel database. For statistical analysis SPSS v. 10 was used and for calculation of statistical significance between demographic groups Pearson’s $\chi^2$ test was applied. Level of statistical significance was set at $p \leq 0.05$.

RESULTS

Frequency of *P. ginseng* and its analogues use

Of all study participants, 18.1% ($n = 223$) reported the use of *P. ginseng* of whom 49 respondents (4.0%) in addition consumed *P. ginseng* analogues in their lives (Table 1). Of the users, 48.9% ($n = 109$) had used *P. ginseng* preparation up to four years prior to the study, 31.4% ($n = 70$) five to ten years and 6.7% ($n = 15$) more than ten years ago. Described distribution could be seen as a signal about relatively recent popularity of *P. ginseng* in Estonia.

Of the *P. ginseng* users, 67.3% ($n = 150$) reported single and 32.7% ($n = 73$) repeat use of this medicinal plant. These results demonstrate rather medium interest towards *P. ginseng* preparations. However, the repeat use of *P. ginseng* preparations could be connected with positive effect of these preparations as only five study participants (6.8%) reported not having any effect and 30.1% ($n = 22$) could not determine the positive effect in case of multiple use of *P. ginseng*.

Duration of the use might also contribute to perceived satisfaction of selected preparation. Of the *P. ginseng* users, 38.6% ($n = 86$) consumed these preparations for more than one month during one course of the treatment, 35.4% ($n = 79$) for 2-3 weeks, 19.3% ($n = 43$) for less than a week and 6.7% ($n = 15$) claimed using *P. ginseng* more or less constantly.

The most popular of the three proposed species of *P. ginseng* analogues was *E. senticosus* (indicated 32 times). Rather popular was also *R. rosea* L. (22 times). To some extent *S. chinensis* was used less (named 5 times). Of the users of *P. ginseng* analogues 14.3% ($n = 7$) had used two and only 4.1% ($n = 2$) all three species of analogues.

Demographic data of non-users and users of *P. ginseng* were compared. With increase of age and among senior residents (both $p < 0.01$), people speaking Russian as native language ($p = 0.02$) and with lower levels of education ($p = 0.04$), the use of *P. ginseng* was less frequent. On the other hand, the use of *P. ginseng* was more common amongst working people and those with university degree and with better self-evaluated health ($p = 0.003$). Described results could be explained with reduced need for stimulants in older age and possible lack of awareness about exotic remedy among people with lower levels of education. More frequent use of *P. ginseng* in

![Figure 2. Self-evaluated health and perceived positive effect of *P. ginseng* preparations](image-url)
Tartu (known as a town with one of the oldest universities in Northern Europe) could be connected with higher number of inhabitants with university degree.

Of the 212 respondents which answered to the question, 75.0% (n = 159) claimed the use of only one specific Panax ginseng preparation and 18.4% (n = 39) claimed the use of more than one specific preparation.

### Table 1. Socio-demographic characteristics of non-users and users of Panax ginseng.

<table>
<thead>
<tr>
<th></th>
<th>Non-users n = 1010</th>
<th>Users n = 223</th>
<th>Statistical significance (p &lt; 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16-30</td>
<td>250</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>31-45</td>
<td>269</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>46-60</td>
<td>205</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>61-75</td>
<td>202</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>75+</td>
<td>84</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>665</td>
<td>158</td>
<td>70.9</td>
</tr>
<tr>
<td>Male</td>
<td>345</td>
<td>65</td>
<td>29.1</td>
</tr>
<tr>
<td><strong>Education</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elementary school</td>
<td>43</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td>High school</td>
<td>228</td>
<td>39</td>
<td>17.5</td>
</tr>
<tr>
<td>Vocational education</td>
<td>288</td>
<td>70</td>
<td>31.4</td>
</tr>
<tr>
<td>University degree</td>
<td>451</td>
<td>112</td>
<td>50.2</td>
</tr>
<tr>
<td><strong>Native language</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>621</td>
<td>158</td>
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</tr>
<tr>
<td>Russian</td>
<td>389</td>
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<td>29.1</td>
</tr>
<tr>
<td><strong>Occupation</strong></td>
<td></td>
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<tr>
<td>Working</td>
<td>544</td>
<td>157</td>
<td>70.4</td>
</tr>
<tr>
<td>Student</td>
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<td>16</td>
<td>7.2</td>
</tr>
<tr>
<td>Senior</td>
<td>267</td>
<td>29</td>
<td>13.0</td>
</tr>
<tr>
<td>Unoccupied</td>
<td>61</td>
<td>6</td>
<td>2.7</td>
</tr>
<tr>
<td>Working student</td>
<td>43</td>
<td>8</td>
<td>3.6</td>
</tr>
<tr>
<td>Working senior</td>
<td>14</td>
<td>7</td>
<td>3.1</td>
</tr>
<tr>
<td><strong>Self-graded health</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very good</td>
<td>167</td>
<td>24</td>
<td>10.8</td>
</tr>
<tr>
<td>Good</td>
<td>458</td>
<td>107</td>
<td>48.0</td>
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<tr>
<td>Average</td>
<td>230</td>
<td>73</td>
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</tr>
<tr>
<td>Satisfactory</td>
<td>134</td>
<td>17</td>
<td>7.6</td>
</tr>
<tr>
<td>Unsatisfactory</td>
<td>21</td>
<td>2</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>Place of interview</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tartu</td>
<td>296</td>
<td>73</td>
<td>32.7</td>
</tr>
<tr>
<td>Kohila-Järve</td>
<td>297</td>
<td>43</td>
<td>19.3</td>
</tr>
<tr>
<td>Tallinn Töömäe</td>
<td>202</td>
<td>37</td>
<td>16.6</td>
</tr>
<tr>
<td>Tallinn Mustamäe</td>
<td>141</td>
<td>12</td>
<td>5.4</td>
</tr>
<tr>
<td>Tallinn Pirita</td>
<td>297</td>
<td>58</td>
<td>26.0</td>
</tr>
</tbody>
</table>
39) at least two different preparations. The rest of the respondents did not use preparations showed to them on the illustration or did not remember the specific preparations they used. Among the depicted preparations (Fig. 1) the most popular were tablets and solution containing P. ginseng as single component (no. 1-3) all together named 89 times and in combination with vitamins and minerals (no. 5), 13 times (Fig. 1). The pattern of those respondents reported only the use of mono-component P. ginseng preparations was similar to the all users of P. ginseng.

Selected preparations signal that a majority of the respondents reporting the use of P. ginseng, preferred to take tablets. The popularity of tablets was also confirmed by the parallel question addressing the preferred form of preparation, where tablets or capsules were used more frequently by 57.0% (n = 127) of the respondents and tinctures or liquid extracts by 20.2% (n = 45) of the respondents. More than half of the respondents, who had used tablets and capsules, reported positive effects of P. ginseng (p = 0.032).

**Purpose of use and perceived effects of P. ginseng and its analogues**

Of the respondents which used P. ginseng, 57.8% (n = 129) used those preparations for one and the rest for multiple indications. More popular single indications were general stimulation - 22.9% (n = 51), increase of mental capacity 17.0% (n = 38) and immune-stimulation 13.0% (n = 29). In addition, health recovery, increasing of appetite and blood pressure and antidepressant activity were mentioned by only few respondents. Of the multiple applications more popular were the combinations of general stimulation and an increase of mental capacity 13.5% (n = 30).

Despite whether P. ginseng preparations were used for single or multiple indications, only 46.2% (n = 103) of the respondents claimed that their health problem was solved; while 15.7% (n = 35) study participants reported that there was no effect. The rest of the respondents 38.1% (n = 85) could not evaluate if the P. ginseng preparation was effective or not.

Respondents who considered their health as very good, good or average experienced more positive effect of the P. ginseng intake than those with satisfactory or unsatisfactory self assessment of their health condition (p < 0.01) (Fig. 2). Rather surprisingly, residents of Tallinn experienced less positive effect of P. ginseng intake than residents of Tartu and Kohtla-Järve (p = 0.03). This may be related to speed-up living in Tallinn, the capital city of Estonia and hence probable higher expectations to a “miracle” medicine.

Of the P. ginseng analogue users, more than half (57.1%) claimed positive outcome of the treatment. The rest was not able to comment on the influence of the P. ginseng analogues on their health 34.7% (n = 17) or they did not experience any effect of its analogues 8.2% (n = 4).

**Side-effects of P. ginseng preparations**

Of the study participants used P. ginseng, 13.5% (n = 30) reported side effects. More frequent problems were 4.5% increased blood pressure (n = 10) and gastrointestinal tract irritation 3.6% (n = 8), but also anxiety 1.8% (n = 4), insomnia, arrhythmia and nasal bleeding 0.5% (n = 1). One respondent (0.5%) had experienced several side effects.

**DISCUSSION**

This is the first study in Estonia exploring the use for self-treatment and knowledge about exotic medicinal plants – P. ginseng and its analogues. The study results serve as a good example of newly introduced and widespread herbal product in Northern Europe.

Only few international studies have evaluated the frequency in the use of P. ginseng preparations. In those studies the number of respondents who have used P. ginseng within specified period of time varies within a wide range and is dependent on demographic characteristics. A study conducted among Sikh (Punjabi) community in London found the rate of citation to be 12% (11). This is still in times higher than in a study conducted in USA, where Cherniack and co-authors (12) found that 5% of the subset of respondents used P. ginseng preparations for self-treatment and the use rate among white population was even below 1%.

The high proportion of the users of P. ginseng detected in this study could be explained by covering the life-time period and questioning the respondents at the pharmacies, not covering the part of the population that do not attend pharmacies regularly. In Northern Europe P. ginseng combined with minerals was found to be particularly popular among Swedish women (6), but such preference was not confirmed by this study.

Priority given to tablets could be explained as well with the ease of taking ones, but also that tablets are culturally accepted form of medicine in Estonia for already longer period of time. Although the intake of liquids in form of medicinal teas is...
rather common in Estonia (16), the intake of tinctures containing alcohol appears to be of less cultural importance. It could be explained with tinctures and infusions contain most often alcohol, which can influence the ability to drive, although the quantities are small.

In this study, the popularity of Panax ginseng and its perceived effects as a stimulant is worthy of attention, demonstrating rather well defined position of the Panax ginseng preparations within the herbal landscape of Estonians. The use of stimulants in Estonian traditional medicine is not common or at least it was not perceived as such, although immune-stimulation could be seen as one of the reasons to use medicinal plants for prevention of cold and cold relate diseases, which was rather popular already in 19th century (17, 22).

The perception of the study participants on the safety and the side effects of ginseng corresponds with former international research that has described Panax ginseng as herbal preparation with a good safety profile and low incidence of adverse effects (30) and a double-blind, placebo-controlled, parallel group clinical study reported mild adverse events as dyspepsia, hot flash, insomnia and constipation in both placebo and Panax ginseng group subjects (31).

Study limitations

The study did not include the entire population, but only the samples of pharmacy customers in three different regions of Estonia. However, community pharmacies are the most frequent places where one can purchase Panax ginseng preparations in Estonia and selected study sample and setting created the foundation for collecting the reliable information.

The selection of illustrated Panax ginseng preparations was limited to those available at community pharmacies at the time of the study. This could be the reason why the participants who had used Panax ginseng preparations more than five years ago did not recall correctly the used preparation.

CONCLUSIONS

This study gives initial information about lay perception and use of Panax ginseng and its analogues, based on the sample of pharmacy customers in North-European country Estonia. Based on the results, the sample customer of Panax ginseng is a middle aged (31-60 years) Estonian female with university degree and a good health.

In comparison with other studies and considering the region, the use of Panax ginseng was rather frequent – about 1/5 of the study participants. Panax ginseng preparations were used mostly according to the well-known indications - tiredness, weakness and decreased mental and physical capacity. Thus, Panax ginseng could be seen as a helping tool in the treatment of such conditions, where the use of traditional medicinal plants of Estonia has not been well-established (e.g., mental stimulation).

The efficacy of Panax ginseng and its analogues was evaluated similarly – about half of the users experienced positive effects, 1/3 could not determine the effects and the rest did not experience any improvement of their condition. Described outcomes support the results of international studies about inconsistency of Panax ginseng efficacy. On the other hand, evaluation of the efficacy of analogues on the similar level with more studied Panax ginseng demonstrate existing practical experience of Estonian pharmacy customers about the use of different exotic medicinal plants.

Although the study gives a short overview concerning the frequency and content of side effects experienced by Panax ginseng users, the future research has to concentrate more on the safety aspects (interactions, contraindications) of Panax ginseng and its analogues.

Acknowledgments

The authors are indebted to M.Sc. pharm. Anna Abolinö and M.Sc. pharm. Maanus Raud for data collection and initial data analysis.

REFERENCES


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In order to combat diseases throughout the world, drug substances as well as vaccines are considered as vital remedies, but at the same time, these may also cause severe adverse drug reactions that depend upon specific factors related to patient as well as product. The fact is further imperative in low economy countries, whereby a majority of the medicines can be purchased without any prescription, including drugs associated with higher frequency of adverse effects (1, 2).

When related to a bigger tendency in term of self-medication, unauthorized drug use can impart troublesome outcomes for public health in developing countries. Most surveys demonstrate that prevalence of self medication might be in the range of 6.3-51.3% that mainly depends upon the location (3, 4).

Nevertheless, such type of self-medication with suitable supervision is sometimes advantageous, as the people have inadequate access to health care facilities in countries having low income and lack of resources. In this perspective, pharmacy function has been extended by adding not merely dispensing activities, but also health care education, and in some cases diagnostic activities.

In the developing countries, personnel involved in drug selling in the unofficial sectors are frequently considered as the initial mean of accessing health care; particularly in setups having self-medication is on its peak (4). Those, who are involved in self-medication, have mentioned reasons responsible for self medication feasibility, convenient in nature, efficacious, dependability of supply and decreased charges (5). Unluckily, most of the drug sellers are practicing to advise medicines with profitable price margins irrespective of any concern related to the suitability (e.g., efficacy, indications, safety) of the drugs and without having any professional qualification (6).

An initial study carried out in Pakistan demonstrated that numerous pharmacy proprietors have nominal academic qualification and negligible or no professional training; of persons properly trained in their profession, nearly all were not present in their pharmacies (7), a trend also found in other developing countries (2, 5, 8-10). Having inadequate understanding of drug sellers concerning indications, contra-indications, and adverse events of drugs, their dispensing practices may lead to redundant effects.

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**ASSESSMENT OF PRACTICE AT RETAIL PHARMACIES IN PAKISTAN: EXTENT OF COMPLIANCE WITH THE PREVAILING DRUG LAW OF PAKISTAN**

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**Abstract:** The main objective of this study was to assess the practice at retail pharmacies in Pakistan and to compare the same in rural and urban areas. The maintenance of pharmacy and drug inspectors’ visit was also assessed. This cross sectional study was conducted in Abbottabad, Pakistan during October-November, 2012. A sample of 215 drug sellers or drug stores was selected by employing convenient sampling method. With a response rate of 91.6%, 197 drug sellers participated in this study. All the drug sellers were male. Overall, 35% (n = 197) of the drug sellers did not have any professional qualification. A majority of the drug sellers were involved in various malpractices like selling of medicines without prescription (80.7%), prescribing practice (60.9%), prescription intervention (62.4%) and selling of controlled substances (66%) without a license for selling it. These malpractices were significantly higher in rural area than that in urban area.

**Keywords:** retail pharmacy, drug inspectors, pharmacy practice

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Table 1. Demographic data of the participants (n = 197).

<table>
<thead>
<tr>
<th>Age (Mean ± SD)</th>
<th>37.13 ± 9.62</th>
</tr>
</thead>
<tbody>
<tr>
<td>Years of experience (Mean ± SD)</td>
<td>10.58 ± 6.45</td>
</tr>
<tr>
<td><strong>Locality</strong></td>
<td></td>
</tr>
<tr>
<td>Rural (%)</td>
<td>35</td>
</tr>
<tr>
<td>Urban (%)</td>
<td>65</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
</tr>
<tr>
<td>Male (%)</td>
<td>100</td>
</tr>
<tr>
<td>Female (%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Professional qualification of drug sellers.

<table>
<thead>
<tr>
<th>Qualification</th>
<th>Frequency</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharm. D.</td>
<td>27</td>
<td>13.7</td>
</tr>
<tr>
<td>Diploma in pharmacy</td>
<td>41</td>
<td>20.8</td>
</tr>
<tr>
<td>Category C holder</td>
<td>29</td>
<td>14.7</td>
</tr>
<tr>
<td>Diploma in health technology</td>
<td>25</td>
<td>12.7</td>
</tr>
<tr>
<td>Currently a student</td>
<td>6</td>
<td>3.0</td>
</tr>
<tr>
<td>None</td>
<td>69</td>
<td>35.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>197</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 3. Comparison of various malpractices in rural and urban area.

<table>
<thead>
<tr>
<th></th>
<th>Total % (n = 197)</th>
<th>Comparison of rural and urban area</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selling medicines without prescription</td>
<td>80.7</td>
<td>95.7</td>
<td>72.7</td>
</tr>
<tr>
<td>Involvement in prescribing practice</td>
<td>60.9</td>
<td>71.0</td>
<td>55.5</td>
</tr>
<tr>
<td>Selling of controlled substances</td>
<td>66.0</td>
<td>63.8</td>
<td>67.2</td>
</tr>
<tr>
<td>Involvement in prescription intervention</td>
<td>62.4</td>
<td>72.5</td>
<td>57</td>
</tr>
</tbody>
</table>

NS = not significant

Table 4. Cross tabulation of locality × frequency of drug inspectors’ visit.

<table>
<thead>
<tr>
<th>Locality</th>
<th>Frequency of drug inspectors’ visit</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Once per month</td>
<td>Once in 3 months</td>
</tr>
<tr>
<td>Urban</td>
<td>Count</td>
<td>59</td>
</tr>
<tr>
<td>Rural</td>
<td>Count</td>
<td>3</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>Count</td>
<td>62</td>
</tr>
</tbody>
</table>
The Government of Pakistan has defined conditions for the issuance of a license for sale of drug at retail level. The drug law requires that premise of pharmacy must possess suitable and adequate facilities for the storage of drug substances. Moreover, premises should be in clean and hygienic conditions. Drug should be sold under continuous personal supervision of a registered pharmacist. Furthermore, the Government of Pakistan appoints drug inspectors, who are responsible for inspection of premises where drug are stored, sold or offered for sale. Also, these drug inspectors are responsible for seizure of drugs and sealing of premises, when drug are stored, sold or offered for sale in contravention of the Drug Act, 1976 (11).

Provided that Pakistan has inadequate statistics on the operation of retail as well as whole sale pharmacies, this study was conducted to assess the adherence of retail pharmacies to prevailing drug law of Government of Pakistan.

**METHODOLOGY**

This cross sectional study was conducted in Abbottabad, Pakistan during October - November, 2012. The methodology for data collection was adopted from some previous studies (12, 13). A sample of 215 drug sellers of drug stores was selected by employing convenient sampling method. The drug sellers were interviewed through pre-tested self administered questionnaire after explaining them the purpose of the study. Furthermore, the participants were assured about the confidentiality of data provided by them. The questionnaire consisted of two parts. First part contained demographic queries. The second part consisted of various questions.

<table>
<thead>
<tr>
<th>Table 5. χ² tests for locality × frequency of drug inspectors’ visit.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>Pearson χ²</td>
</tr>
<tr>
<td>Likelihood ratio</td>
</tr>
<tr>
<td>Linear-by-linear association</td>
</tr>
<tr>
<td>Number of valid cases</td>
</tr>
</tbody>
</table>

d.f. = degree of freedom; Asymp. sig. = asymptotic significance value

<table>
<thead>
<tr>
<th>Table 6. Cross tabulation of locality × maintenance of pharmacy.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maintenance of pharmacy</td>
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<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Poor</td>
</tr>
<tr>
<td>Urban</td>
</tr>
<tr>
<td>Rural</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 7. χ² tests for locality × maintenance of pharmacy.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Pearson χ²</td>
</tr>
<tr>
<td>Likelihood ratio</td>
</tr>
<tr>
<td>Linear-by-linear association</td>
</tr>
<tr>
<td>No. of valid cases</td>
</tr>
</tbody>
</table>

d.f. = degree of freedom; Asymp. sig. = asymptotic significance value
regarding the possession of license, prescribing practice of medicines, selling of medicines without prescription, selling of controlled substances, maintenance of pharmacy and involvement in prescription intervention. Frequency of drug inspector’s visits was also investigated through the questionnaire.

Statistics

Data were analyzed through SPSS version 17.0. The level of significance was kept at 0.05.

RESULTS AND DISCUSSION

With a response rate of 91.6%, 197 drug sellers participated in the study. Out of these participants 128 were drug sellers of urban area whereas 69 were of rural area. The mean age of the participants was 37.13 ± 9.62 years and mean duration of experience was 10.58 ± 6.45 years.

The professional qualification of the participants has been shown in Table 1. Out of 197, 13.7% were pharmacy graduates. Another 20.8% were diploma holder in pharmacy education, while 14.7% were registered internees, who can work only under the supervision of registered pharmacist but cannot perform independent practice. Alarming, 35% of the participants did not have any professional qualification. Thus, drug seller without professional qualification is a potential menace to public health by dispensing wrong medications to the patients especially in case when they are involved in prescribing practice and prescription intervention. Furthermore, without having any knowledge of correct storage conditions for drugs including vaccines and other biologics may pose the medicines to harsh environment, which may thus cause deterioration of the drugs, leading not only to loss in the potency of the drugs but also produce harmful degradation products (4).

Table 2 shows that 62.4% of the participants were having license for retail sale, whereas 5.6% of them were license holder for wholesale. Another one fourth of the total drug sellers (26.9%) were those having no license at all. Moreover, only 5.1% of the participants possessed license for the sale of controlled substances. But a total of 66% drug sellers were involved in selling of controlled substances. However, drug sellers of rural and urban area were equally involved in the sale of controlled substances and there was no significant difference between drug sellers of rural and urban area regarding the sale of controlled substances without license for selling it (Table 3). As mentioned in Table 3, a total of 80.7% of drug sellers (rural = 95.7%, urban = 72.7%) were involved in malpractice of selling medicines without prescription, as refusal of a patient’s request may cause loss in business and decrease in profit. This malpractice was significantly higher in rural area as compared to urban area (p = 0.0001). Also the prescribing practice of drug sellers was significantly higher in rural area than that in urban one (p = 0.0285). The possible reason for such types of malpractice is lack of primary health care facilities in far flung areas, where public has to seek medical advice from the drug sellers (11). Also, pharmacy setup is easily accessible by patients and is less socially distant than other health care providers, including physicians. Nevertheless, these types of malpractices should be discouraged as these contravenes the correct practices and are the potential sources of self medication, drug misuse and abuse, drug addiction and related adverse drug events (2).

Moreover, malpractice of prescription intervention by drug sellers was significantly higher in the rural area as compared to urban area (p = 0.0323). As in Pakistan, medicines are prescribed by brand names; unavailability of brands prescribed by physicians in rural area could be the possible reason responsible for higher proportion of prescription intervention by drug sellers of rural area as the drug sellers do not want to refuse customer’s request as this may lead to loss in business and decrease in profit (12).

As evident from Tables 4 and 5, the frequency of drug inspectors’ visit is less frequent in rural area as compared to urban area. There is a strong association between locality and frequency of drug inspectors’ visit (p = 0.000). From Tables 6 and 7, it is concluded that the maintenance of pharmacy is more unsatisfactory in rural area than that in urban area. An association between locality and maintenance of pharmacy has been found by using Pearson χ² test (p = 0.000). Over all the poor maintenance at retail pharmacies, rural area is due to the lack of check on retail pharmacies in rural area as compared to urban area as drug inspectors do not visit the former more frequently. There is one drug inspector for each district in the whole province. Thus, it is difficult for the drug inspector to check retail pharmacies in the whole district due to overburden of duty (12). In order to discourage such types of malpractice, the folk should be properly educated about self medication and its consequences as education is more promising for long term change than done by the enforcement of legislation. Moreover, the number of drug inspectors should be increased in order to ensure proper visit and check the maintenance and status of the retail pharmacies.
CONCLUSION

It is concluded from our findings that a majority of the drug sellers do not have the required professional qualification. In addition, these drug sellers are involved in selling of medicines without prescription, prescribing practice, prescription intervention and selling of controlled substances without a license for selling it. Moreover, many pharmacies were not maintained properly. The drug inspectors do not frequently visit the retail pharmacies, possibly due to overburden of duty.

REFERENCES


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