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REVIEW

GENUS *RUELLIA*: PHARMACOLOGICAL AND PHYTOCHEMICAL IMPORTANCE IN ETHNOPHARMACOLOGY

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Abstract: *Ruellia* is a genus of flowering plants commonly known as *Ruellias* or Wild Petunias which belongs to the family Acanthaceae. It contains about 250 genera and 2500 species. Most of these are shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions. They are distributed in Indonesia and Malaysia, Africa, Brazil, Central America and Pakistan. Some of these are used as medicinal plants. Many species of the genus has antinociceptive, antioxidant, analgesic, antispasmolytic, antiulcer, antidiabetic and anti-inflammatory properties. The phytochemicals constituents: glycosides, alkaloids, flavonoids and triterpenoids are present. The genus has been traditionally claimed to be used for the treatment of flu, asthma, fever, bronchitis, high blood pressure, eczema, and diabetes. The objective of this review article is to summarize all the pharmacological and phytochemical evaluations or investigations to find area of gap and endorse this genus a step towards commercial drug. Hence, further work required is to isolate and characterize the active compounds responsible for these activities in this plant and bring this genus plants to commercial health market to serve community with their potential benefits.

Keywords: phytochemical constituents, biological, *Ruellia*, Acanthaceae

Plants are being used as medicines since the beginning of human civilization; healing powers are reported to be present in plants and therefore, it is assumed that they have medicinal properties. The flora of Pakistan due to its diverse climate, soil conditions and many ecological regions is very rich in medicinal plants. According to a survey of Pakistan, about 6000 species of flowering plants have been existing, out of 6000 about 400-600 are medicinally important species (1). From near past, it has been discovered that properties of medicinal plants are due to its active chemical compounds (2). The discovery of drugs from medicinal plants started from the era when the isolation of drugs such as digitoxin, quinine, cocaine, and codeine has begun. The family Acanthaceae (Acanthus family) is a taxon of dicotyledonous flowering plants containing almost 250 genera and about 2500 species. Most of these are tropical herbs, shrubs, or twining vines; some are epiphytes. Only a few species are distributed in temperate regions. Indonesia, Malaysia, Africa, Brazil and Central America are the main producers. *Ruellia* is a genus of flowering plants commonly known as *Ruellias* or wild petunias (1). It is a genus of 250 species, distributed in tropical and temperate regions of both the hemispheres, which is also present in Pakistan by 5 species, of which 3 are native (3).

Present review paper summarizes all the information relating to biological, phytochemical constituents and folk medicinal uses of genus *Ruellia*. These are popular ornamental plants. Some of them are used as medicinal plants. One of the species, *Ruellia tuberosa* has been extensively used as diuretic, anti-diabetic, antipyretic, analgesic, antioxidant (4), anti-hypertensive, gastroprotective (5) and to treat gonorrhea (3). The phytochemical properties of *Ruellia tuberosa* has been investigated revealing the presence of alkaloids, triterpenoids, saponins, sterols and flavonoids (6). *Ruellia asperula* was used in bronchitis, asthma, flu, fever and uterus inflammation (7). *Ruellia prostrate* leaf is used in the treatment of chronic rheumatism, eczema, facial paralysis, cephalgia and hemiplegia. Leaf juice is an efficient remedy in colic of children (8). The fresh leaf of the plant *Ruellia patula* is pounded and then soaked in water until the solution turns black. It is decanted and the solution applied to the ear (9). *Ruellia hygrophila* has antispasmodic, analgesic activity.

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Taxonomy

The family Acanthaceae, Class Eudicots and order Lamiales includes 250 genera and 2500 species (10). In Pakistan it is represented by 18 genera and 60 specific and infra specific taxa, of which 44 are native (11). The first palynological study was investigated few taxa (12). However, forthcoming studies (13) carried out detailed study and recognized for the first time 11 pollen types within the family Acanthaceae.

Folk medicinal uses of genus *Ruellia*

The whole parts of genus *Ruellia* are used in bladder stones and in bronchitis. Paste of leaves is also used for skin diseases and boils. Roots are used as anthelmintic. Syrup is used for whooping cough. Tuber powder is used for stomach ache. *Ruellia tuberosa* has been extensively used for the treatment of emesis, analgesia, nociceptive pain, inflammation, renopathy and syphilis (14). *Ruellia tuberosa* is used in stomach cancer.

In folk medicine and Ayurvedic medicine the genus *Ruellia* has been used as diuretic, anti-diabetic (15) antipyretic, analgesic, antioxidant (4) antihypertensive, gastroprotective (8) agent and was also used to treat gonorrhea (16).

Scientific studies of genus *Ruellia*

Antioxidant activity

The antioxidant activity of different extracts of stem of *Ruellia tuberosa* were investigated by various *in vitro* methods like 2,2-diphenyl-1-picrylhydrazyl (DPPH), free radical-scavenging assays and the hydrogen peroxide induced luminol chemiluminescence assay. The methanol extract and its four fractions of water, ethyl acetate, chloroform, and *n*-hexane were evaluated for antioxidant activity. The results revealed that *Ruellia tuberosa* possesses potent antioxidant activity (4).

It has been reported by other authors that the aerial parts of plant *Ruellia prostrata* showed antioxidant potential. Different concentrations of methanolic extract and n-butanolic fraction were subjected to antioxidant assay by DPPH method, nitric oxide scavenging activity and reducing power assay. Both the n-butanolic and methanolic extract showed the antioxidant potential but the antioxidant potential of n-butanolic fraction is far higher than the methanolic extract (5).

Gastroprotective and analgesic activity

It was reported that aqueous extract of *Ruellia tuberosa* roots showed a strong and dose dependent gastroprotective activity in alcohol-induced gastric lesion of rats. The extract also had a mild erythropoietic and moderate analgesic activity. It was concluded from the data that *Ruellia tuberosa* root extracts have gastroprotective activity (6).

Anti-ulcer activity

Preliminary ethyl acetate extract of *Ruellia tuberosa* was studied for the acute oral toxicity, according to the economic cooperation and development guidelines, based on which two doses were selected, 250 mg/kg (low dose) and 500 mg/kg (high dose). Ranitidine was used as the standard drug (20 mg/kg). The ethyl acetate extract showed significant decrease in gastric volume, total acidity and free acidity. There was a significant ($p < 0.01$) increase in gastric pH only at high dose (500 mg/kg), when compared to control group. The value of ulcer index decreased in a dose dependent manner, when compared to control group (17).

***In vitro* purgative and cholinergic activity**

Methanolic, ethyl acetate and aquous extracts of *Ruellia tuberosa* produced contractions on electrically induced contracted ileum tissue strip at the dose of 30 µg/mL. Methanolic extract of *Ruellia tuberosa* was investigated on uterus and gestation by using 350 mg/kg/day and was found to increase the number of implantation (8). This study demonstrated that estrogenic effect may be due to flavonoid and sterol while cholinergic effect may be due to iridoid glycosides.

Antimicrobial activity

The antibacterial activities of n-hexane, dichloromethane, ethyl acetate and methanol extracts of *Ruellia tuberosa* were explored against Gram positive and Gram negative bacteria. The ethyl acetate and methanol fractions exhibited the highest rates of antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa* (18).

Anticancer activity

The methanol extract of aerial part of herb *Ruellia tuberosa* possessed cytotoxicity. The minimum inhibitory concentration (IC_{50}) for methanol extract was found to be 3.5 and 1.9 µg/mL in H460 and MDA-MB231 cancer cells, respectively. Tylocrebrine was isolated from *Ruellia tuberosa* through bioassay directed column chromatography and elucidated its anticancer and anti-inflammatory potential (19).

Antinociceptive and anti-inflammatory activity

The ethanol extract of *Ruellia tuberosa* was

evaluated for its antinociceptive and anti-inflammatory properties in experimental mice and rat models. In the hot plate test, the group that received a dose of 300 mg/kg for mice showed maximum time needed for the response against thermal stimuli and maximum possible analgesic was similar to that of diclofenac sodium. The extract at 500 and 250 mg/kg doses showed significant reduction in acetic acid-induced writhing in mice, which was similar to diclofenac sodium. The extract also demonstrated significant inhibition in serotonin and egg albumin-induced hind paw edema in rats at the doses of 100, 200 and 300 mg/kg. The anti-inflammatory properties exhibited by the extract were comparable to that of indomethacin at a dose of 5 mg/kg (20).

Cardiovascular and hypertensive activity

In pharmacological investigation, extracts of *Ruellia brittoniana* and *Ruellia patula* were used for cardiovascular screening .The cardiovascular experiments were carried out *in vitro* and *in vivo*. In i*n vitro* studies rabbit heart was used while for *in vivo,* anesthetized rats were used. In i*n vivo* experiment, extracts of *Ruellia brittonana* and *Ruellia patula* showed a hypertensive activity in pentothal sodium anesthetized rats (21).

Antispermatogenic activity

Aqueous extract of tuberous roots of *Ruellia tuberosa* administered orally at the dose of 50, 100 and 150 mg/kg body weight for 21 days resulted in significantly decreased sperm count in male albino rats. The results suggested that the aqueous extract of *R. tuberosa* produces antispermatogenic effect in male albino rats (22).

Antidiabetic, antihyperlipidemic and hepatoprotective activity

The methanol extract of *Ruellia tuberosa* leaves at a dose of 100 and 200 mg/kg of body weight was administered at single dose per day to diabetes-induced rats for a period of 14 days. The methanol extract of *Ruellia tuberosa* leaves elicited significant reductions of blood glucose ($p < 0.05$), lipid parameters except HDL-C, serum enzymes and significantly increased HDL-C at the dose of 200 mg/kg when compared with the standard drug glibenclamide (5 g/kg). From the above result, it may be concluded that methanol extract of *Ruellia tuberosa* leaves possesses significant antidiabetic, antihyperlipidemic and hepatoprotective effects in alloxan-induced diabetic rats (8).

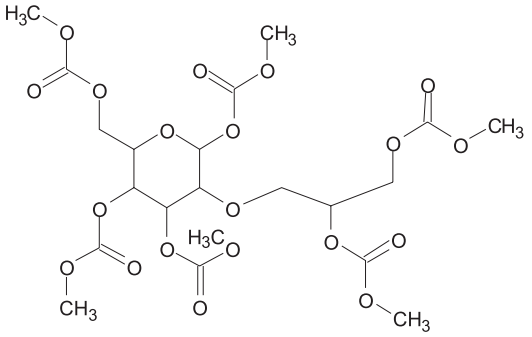
Phytochemical constituents of *Ruellia* genus

Preliminary phytochemical screening

Preliminary phytochemical screening of ethyl acetate extract of *Ruellia tuberosa* reveals the presence of saponins, tannins, and flavonoids, which may be responsible for its activity (18).

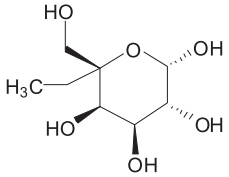
Glycosides

Extract of the whole plant of *Ruellia brittoniana* has afforded the new glycoside 2-O-α-galactopyranosyl glycerol hexaacetate.

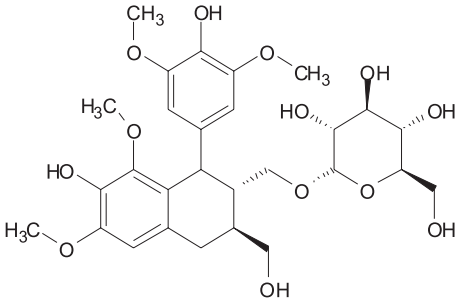


2-O-α-galactopyranosyl glycerol hexacetate

Two lignan glycosides identified as 5,5'-dimethoxylariciresinol-9-α-D-glucopyranoside (reupaside) and lyoniresinol-9'-α-D-glucopyranoside with ethyl-α-D-galactopyranoside, α- and β-D-glucose and β-D-fructose have been isolated from *Ruellia patula* (23, 24).

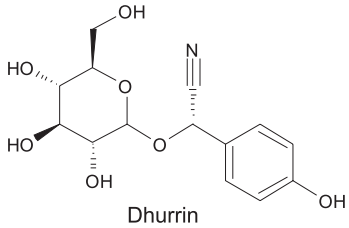


Ethyl- α-D-galactopyranose

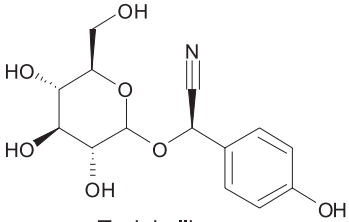


Lyoniresinol-3-β-glucopyranose

A cyanogenic glucoside was isolated from *Ruellia rosea and* showed the presence of a p-hydroxyphenyl moiety indicating that the compound was either taxiphyllin or the diastereomer dhurrin (25, 26).

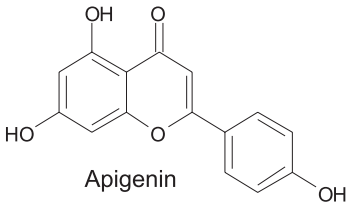


Dhurrin

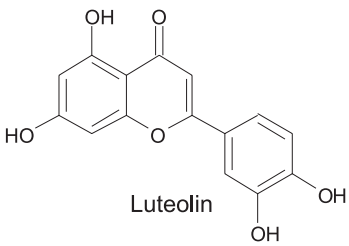


Taxiphyllin

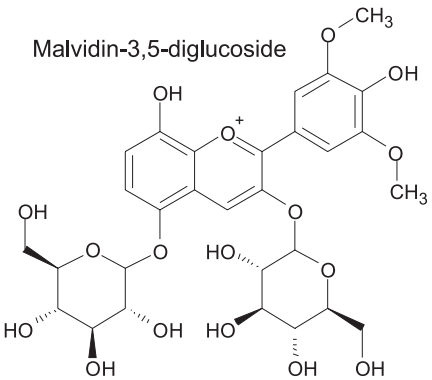
The leaves contained only traces of apigenin and luteolin, while in flowers was malvidin-3,5-diglucoside in appreciable quantity. The flowers buds contained the maximum proportion of flavonoids (3% apigenin-7-O-glucoronide and the other flavones were identified as apigenin-7-O-glucoside, apigenin-7-O-rutinoside and luteolin-7-O-glucoside (26, 27).



Apigenin

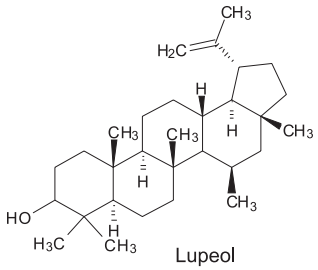


Luteolin

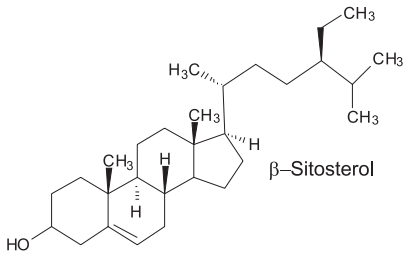


Malvidin-3,5-diglucoside

From *Ruellia patula*, nine compounds were isolated. A new lignan glycoside 5,5-dimethoxylariciresinol-9-O-β-D-glucopyranoside, apigenin-7-O-rutinoside, β-sitosterol, lupeol, α-D-glucose, β-D-glucose, and β-D-fructose (21).



Lupeol

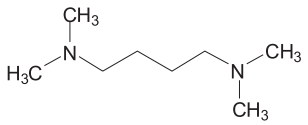


β–Sitosterol

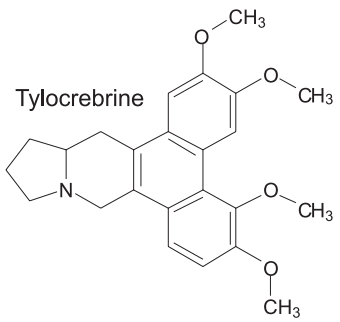
Alkaloids

Tetramethylputrescine was isolated from the roots and aerial parts of *Ruellia rosea* (28).

Tylocrebrine, a phenanthrene alkaloid, was reported from aerial parts of *Ruellia tuberosa* and found its anti-cancer and anti-inflammatory potential (13).



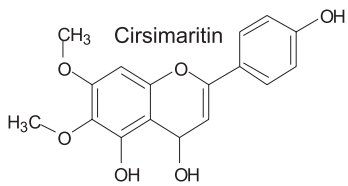
Tetramethylputrescine



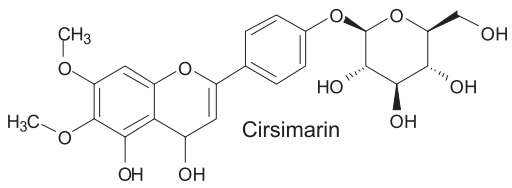
Tylocrebrine

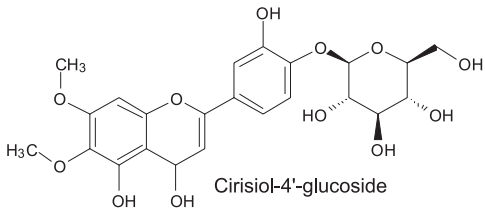
Flavonoids

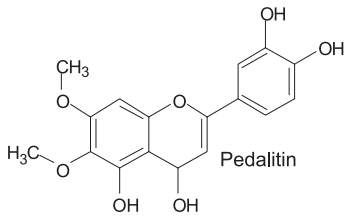
Five flavonoids: cirsimaritin, cirsimarin, cirsiliol-4'-glucoside, sorbifolin and pedalitin along with betulin, vanillic acid and indole-3-carboxaldehyde were isolated from the ethyl acetate extracts of *Ruellia tuberosa* (14).

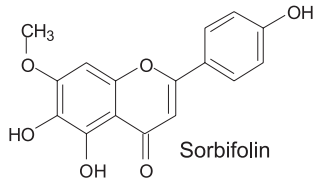


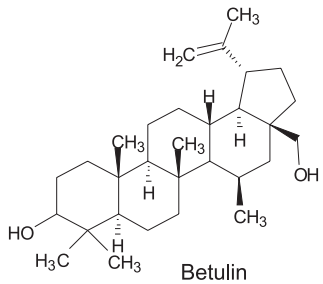
Cirsimaritin

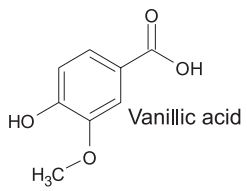


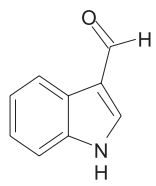










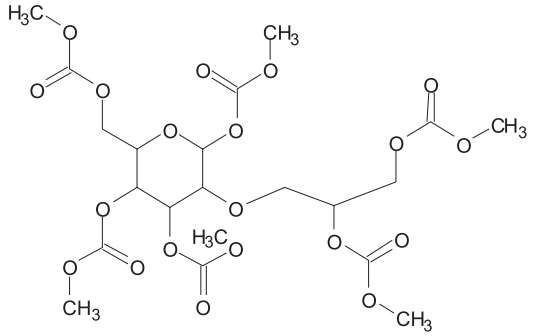


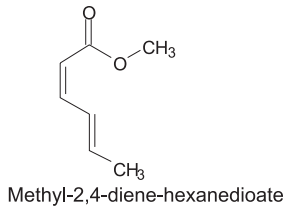
Three new flavonoid glycosides: demethoxycentaureidin 7-O-β-D-galacturonopyranoside, pectolinarigenin 7-O-α-L-rhamnopyranosyl-(1’’’,4’’)-β-D-glucopyranoside and 7-O-α-L-rhamnopyranosyl-(1’’’,4’’)-β-D-glucuronopyranoside, a new megastigmane glucoside, byzantionoside B 6’-O-sulfate, and a new (Z)-hex-3-en-1-ol O-β-D-xylopyranosyl-(1’’,2’)-β-D-glucopyranoside, were isolated from leaves of *Ruellia patula* JACQ., together with 12 known compounds, β-sitosterol glucoside, vanilloside, bioside (decaffeoylverbascoside), acteoside (verbascoside), syringin, benzyl alcohol O-β-D-xylopyranosyl-(1’’,2’)-β-D-glucopyranoside, cistanoside E, roseoside, phenethyl alcohol O-β-D-xylopyranosyl-(1’’,2’)-β-D-glucopyranoside, (+)-lyoniresinol 3-α-O-β-D-glucopyranoside, isoacteoside and 3,4,5-trimethoxyphenol O-α-L-rhamnopyranosyl-(1’’,6’)-β-D-glucopyranoside. Their structures were elucidated by means of spectroscopic analyses.

Other compounds

A triterpenoid, 21-methyldammer-22-en-3,18,27-triol was isolated from the aerial parts of *Ruellia tuberosa* (30).

From *Ruellia brittoniana*, five compounds of different classes were isolated. Two new compounds: 2-O-α-D-galactopyranosyl glycerol hexaacetate and dimer of methyl-2,4-diene-hexandioate were obtained. In addition to these new compounds, three reported compounds, hyoniresinol-9-O-β-D-glucopyranoside, α-ethyl-galactose and para-methoxybenzoic acid were isolated for the first time from this plant (31).



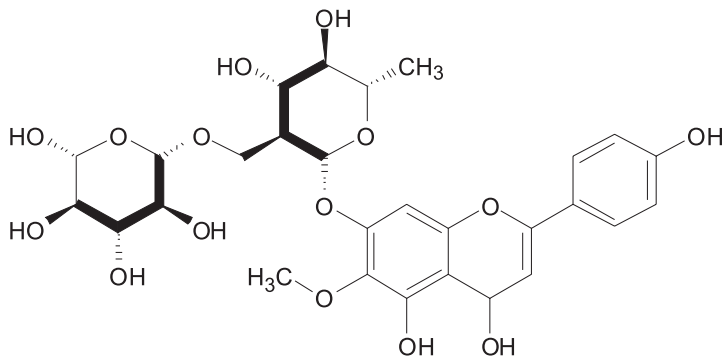


A new phenylethanoid glycoside, is ocassifoliosid, and two new flavone glycosides: hispidulin 7-O-α-L-rhamnopyranosyl-(1’’’,2’’)-O-β-D-glucuronopyranoside and pectolinaringenin 7-O-α-L-rhamnopyranosyl-(1’’’,2’’)-O-β-D-glucuronopyranoside were isolated from the aerial portions of *Ruellia tuberosa*, together with verbascoside, isover-

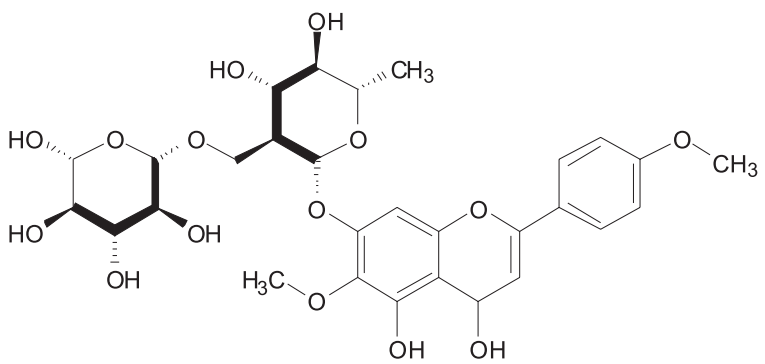
bascoside, nuomioside, isonuomioside and forsythoside B.

CONCLUSION

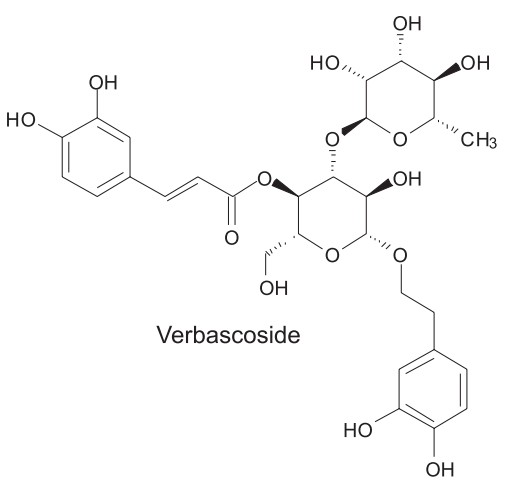
The review place emphasis on genus *Ruellia* and elaborates the biological and phytochemical studies and medicinal uses in various ailments. This genus is well studied in its biological and phytochemical aspects. The future work required is to isolate the active biological compounds which are responsible for these activities and may serve as drugs for different ailments. Now it is time to bring genus *Ruellia* from herbal market to health commercial market.



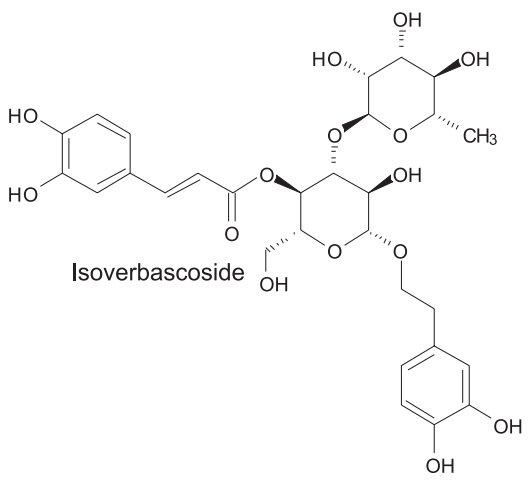
Hispidulin-7-O- α-L-rhamnopyranosyl-(1'-2'')-O- β-D-glucuronopyranoside



Pectolinarigenin-7-O-α-L-rhamnopyranosyl-(1'-2'')-O-β-D-glucuronopyranoside



Verbascoside



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PHARMACOLOGICAL ASSESSMENT OF HISPIDULIN - A NATURAL BIOACTIVE FLAVONE

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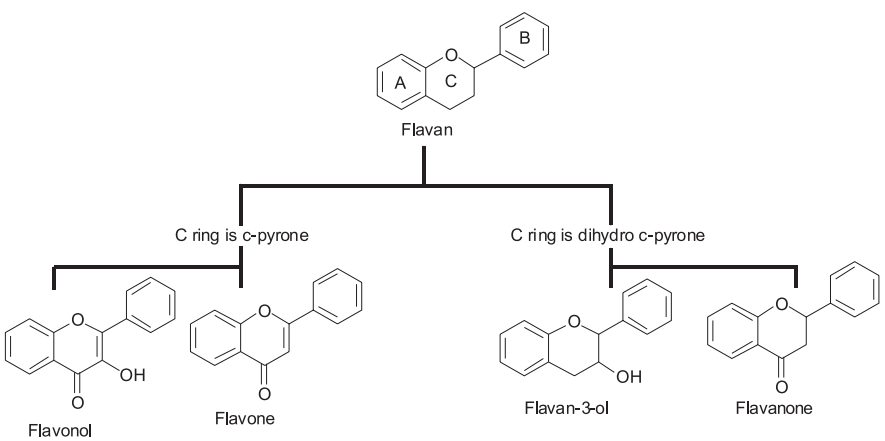
Abstract: Hispidulin is well-known natural bioactive flavone on behalf of its pharmacological aspects. This review contains data on isolation, synthetic methodology, pharmacokinetics and bioactivities of hispidulin. The article provides a critical assessment of present knowledge about hispidulin with some clear conclusions, perspectives and directions for future research in potential applications.

Keywords: antioxidant, anticancer, antiepileptic, antiinflammatory, antiosteoclastogenesis, antihypnotic, hepatoprotective, mitochondrial metabolism

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 $GABA_A$ receptor (2-4).

Flavones are derived from flavan structure (5-7) (see Scheme 1). As displayed in Scheme 1, flavonoids contain a benzene ring (A) condensed with a six membered heterocyclic ring (C) having a phenyl substituent (B) at C-2. The saturation of ring



Sceme 1. Flavonoid classes

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C leads to the flavonol and flavone based structures, in case ring C is c-pyrone, or it might result in flavanol and flavanone if ring C is dihydro derivative of c-pyrone (8). Owing to structural diversity, flavones uncover variety of functions, which not only include biological and pharmaceutical activities (9-17), but also incorporate color control in vegetables and fruits, to protect them from UV radiations as well as infectious attacks of microorganisms (6, 18, 19).

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 (MW) are considered good. PSA of hispidulin has been reported as 100.12 $Å^2$, which is appreciably high with MW 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 $GABA_A$ 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 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 CO_2 *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 un-deteriorated 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 (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 reports 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 testings 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.

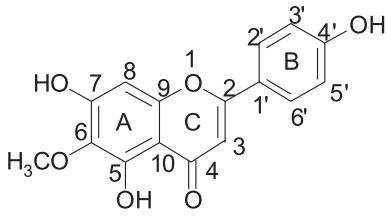


Figure 1. Hispidulin structure

$C_{16}H_{14}O_6$: 302; m.p.: 228-230°C (lit. 115).

UV: 293 (4.23), 331 (3.68); +CH_3ONa, 247 (4.30), 329 (4.44); +CH_3COONa, 294 (4.07), 330 (4.19); +$AlCl_3$, 225 (4.42), 300 sh (4.12), 316 (4.21), 394 (3.41); +$AlCl_3$/HCl, 225 (4.47), 314 (4.32), 394 (3.41) (116)

IR: 3500 (OH), 1640 (C=O γ-pyrone) (lit. 117)

MS: 302 $[M]^+$, 120 C_8H_8O

CD: (c 0.001, MeOH) [θ] (nm): -10744 (300) (negative maximum), +13358 (268) (positive maximum) (lit. 115).

^{1}H-NMR: (DMSO-d_6): 2.80 (1H, dd, *J* = 4.0, H-3 eq), 3.10 (1H, d, *J* = 12.0, H-3 ax), 3.72 (3H, s, OCH_3), 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) (lit. 115).

^{13}C NMR (DMSO-d_6): (lit. 56)

C-2	78.4	C-8	95.1	C-4'	157.9
3	42.1	9	157.6	5'	115.2
4	196.8	10	102.0	6'	127.9
5	155.0	1'	129.0	OCH_3	59.9
6	129.0	2'	127.9		
7	159.4	3'	115.2		

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 Me_2SO_4. Second step involved selective demethylation of **2** to get compound **3** by using $AlCl_3$/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 BCl_3/CH_2Cl_2 resulted in hispidulin **13**.

Biological activities of hispidulin

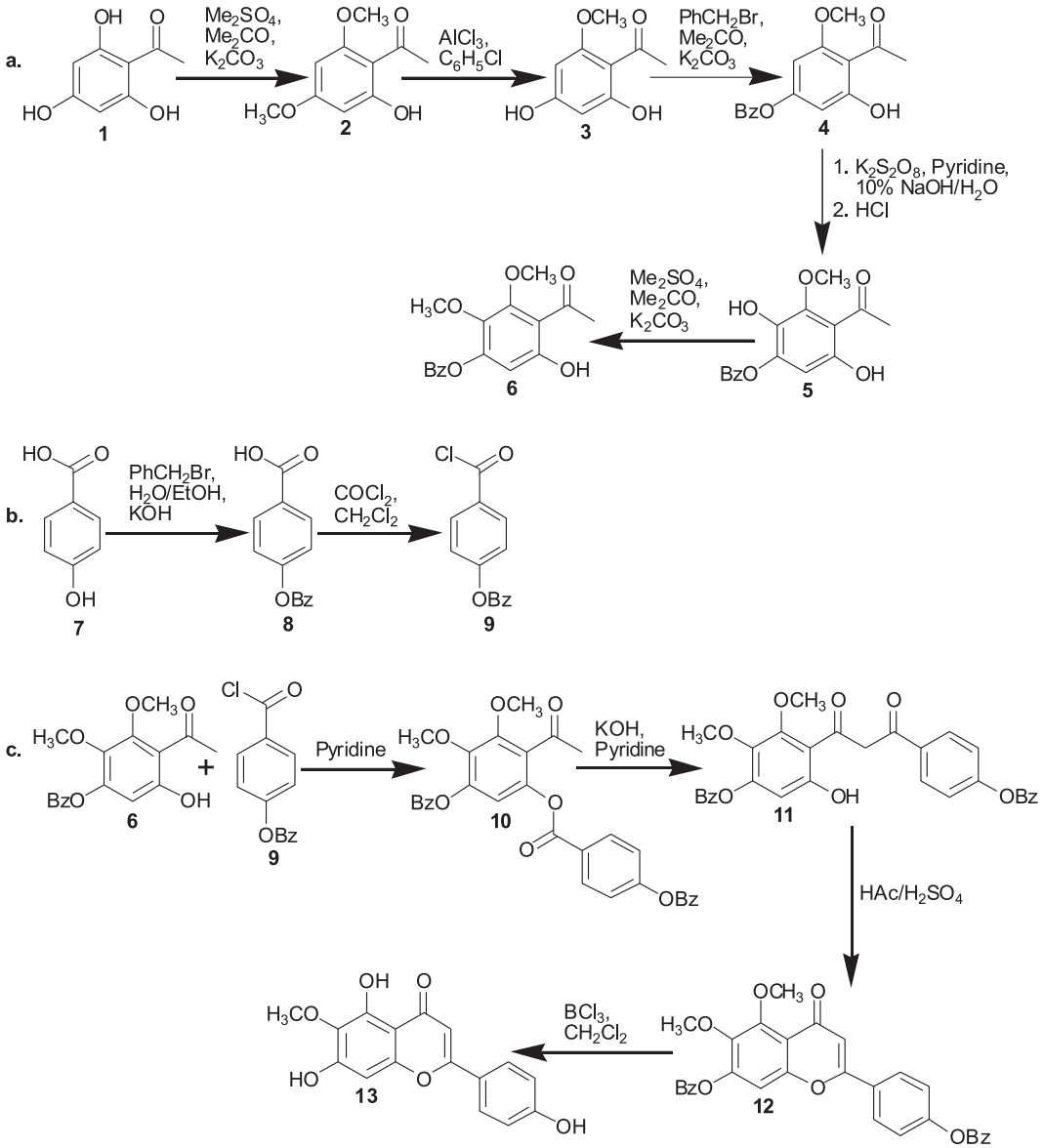
Herbal medication is evolving worldwide, but unsatisfactory certification about their safety or usefulness screens them out. This dilemma, somehow, has been resolved by the improvement and rationalization of appropriate analytical assays. For hispidulin, different pharmacological aspects have been reviewed 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, 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 (1). Molecules that require less energy to break O–H bond, breed stable free radicals and show strong antioxidant behavior.

$$ROH + OH^{\cdot} \rightarrow RO^{\cdot} + HOH$$

Second mechanism narrates transfer of electron from antioxidant to radical species ensuing indirect H-removal. This mechanism depends upon the energy required to craft $ROH^{+\cdot}$ radical cation, through ionization, as well as on the reactivity of radical cation. Normally, flavonoids with low IP are



Scheme 2. Total synthesis of hispidulin through Baker Venkataraman reaction using 4-benzyloxy-2,3-dimethoxy-6-hydroxyacetophenone and 4-benzyloxybenzoic acid chloride

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.

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

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 polarographic 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 mitochondrial swelling. These results correlate mito-

Table 1. Medicinal plants rich in hispidulin.

Compound	Family	Species / Plant	Reference
Hispidulin	Asteraceae	*Centaurea cyanus*	(38)
		Aegialophila pumila	(41)
		Centaurea pichleri	(41)
		Centaurea phyllocephala	(41)
		Centaurea malacitana	(42)
		Centaurea hierapolitana	(47)
		Centaurea phyllocephala	(73)
		Grindelia argentina	(75)
		Centaurea clementei	(117)
		Centaurea furfuracea	(118)
		Centaurea malacitana	(119)
		Centaurea scoparia	(120)
		Cheirolophus mauritanicus	(121)
		Centaurea chilensis	(122)
		Centaurea aspera	(123)
		Centaurea melitensis	(124)
		Centaurea floccosa	(125)
		Centaurea coronopifolia	(126)
		Centaurea phyllocephala	(127)
		Centaurea inermis and C. virgata	(128)
		Centaurea urvillei	(129)
		Centaurea aspera	(130)
		Centaurea arguta	(131)
	Lamiaceae	*Scutellaria repens*	(39)
		Scutellaria baicalensis	(40)
		Salvia officinalis	(43)
		Rosmarinus officinalis	(43)
		Oregano (Folium origani cretici)	(44)
		Sage (Folium salviae officinalis)	(44)
		Thyme (Folium thymi)	(44)
	Compositae	*Artemisia species*	(4)
		Salvia species	(27)
		Arnica montana	(46)
		Centaurea alexandrina	(72)
		Artemisia vestita	(110)
		Centaurea collina	(132)
	Verbenaceae	*Lantana montevidensis* BRIQ.	(56)
Hispidulin 7-O-glycoside	Plantaginaceae	*Plantago asiatica*	(45)
Hispidulin-7-neohesperidoside	Asteraceae	*Cirsium japonicum*	(48, 53)
Hispidulin-7-sulfate		*Centaurea bracteata* (aerial parts)	(49)
Hispidulin-7-sulfate		*Centaurea bracteata* (roots)	(133)

chondrial 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 (5×10^6 each mouse) cancer cells from human pancreas, till tumor extended up to 50 mm^3. 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 IC_{50} 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 IC_{50} value of 20 μM at 72 h treatment in comparison to rutin with IC_{50} value of over 500 μM. Aspirin exhibited minute inhibitory effects on gastric cancer cells (IC_{50} value calculated from graph is over 1 mM). This proves that hispidulin is 25 folds better than rutin and 50 folds better than aspirin, when it comes to their anticancer profile. In the same study, an excessive cyclooxygenase-2 (COX-2) activity in human gastric cancer cells was investigated. The sample cells have been treated with COX-2 inhibitors (celecoxib or NS-398). The observed IC_{50} of celecoxib and NS-398 after 72 h were 30 μM and 40 μM, respectively. Data prove hispidulin as an efficient anti-cancer agent, in comparison to commercial drugs.

A phytochemical study on flavonoids, including hispidulin, from *Rosmarinus officinalis* and *Salvia officinalis*, has been carried out for their anticancer activities (70). Also a constructive connection among antioxidant activity and cytotoxicity was reported (67), concluding both activities as a support either for cancer cell damage or for healthy cell protection during cancer treatment, which attributes to antioxidant exertion. Cytotoxic effect of hispidulin tested against reference anticancer drug - adriamycin, and reported values for hispidulin are less than the reference drug (71). The literature (72) reports a comparison between 30 flavonoids from different plants for their cytotoxic behavior and results indicated that methylation, particularly 6-methylation, augments cytotoxic activity of flavonoids, as in the case of hispidulin. Another research (73) reports the same observations about cytotoxic nature of methylated flavones, in the extracts of *Centaurea phyllocephala* Boiss. The data were collected either by intravenous, intraperitoneal or oral administration of the extracts in unconscious and conscious rats, respectively. The anticancer properties of hispidulin have also been reported (74) in scenario of controlled tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL). In human ovarian cancer cells, hispidulin has been reported to enhance TRAIL-

induced apoptosis, moreover, it is evident from the mechanism elucidation that hispidulin activates caspases 8 and 3 that cleave poly-(ADP-ribose) polymerase (PARP), which is a key factor in programmed cell death. This organized sensitization involved adenosine monophosphate (AMP)-activated protein kinase (AMPK), which is stimulated on treatment with hispidulin. Hispidulin has also been reported (75) to exhibit most potent *in vitro* inhibitory activity against LPS-induced NO production with 98.7% at 50 µg/mL concentration. In addition, hispidulin did not show any effect on cell viability. Primarily, the ethanolic extract of *Grindelia argentina* was found to inhibit the LPS/IFN-γ-induced NO production.

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 ($GABA_A$) receptor activity by 65 ± 17% (4). Stimulation of $GABA_A$ 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 $GABA_A$-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 $GABA_A$ receptor with IC_{50} value of 1.3 µM. A recent study (89) has reported hispidulin as a potent ligand for BZD site of human $GABA_A$ receptor, with 81% inhibition of maximal $GABA_A$ response, showing strongest binding activity to BZD site comparative to ursolic acid, carnosol, oleanolic acid, salvigenin, rosmanol and cirsimaritin. Reported data about hispidulin neuropharmacology, particularly control over epileptic activity through interaction with $GABA_A$ 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_{50} 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

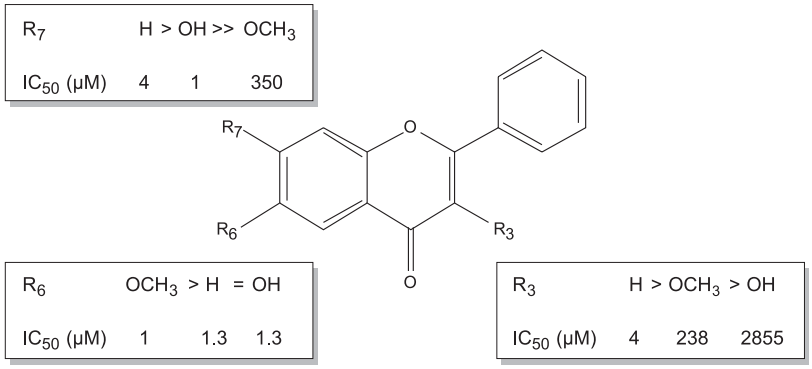


Figure 2. Effect of methoxy substitution on BZD binding affinity

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 10–15% of grownup population (95). Medicinal plants with sedative effect target BZD site of $GABA_A$ 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 $GABA_A$ receptor (96). Hyperpolarization of membrane, by allowing a Cl^- influx, induced by $GABA_A$ 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 $GABA_A$ receptors (99). *In vitro* analysis of different substitutions particularly on flavone structure for their affinity to BZD site of $GABA_A$ 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 galangin-3-methyether (3-methoxy compound) in binding affinity for BZD site of $GABA_A$ receptor. Hispidulin has also been reported from sedative plants with binding affinity value of 8 μM (89).

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.

Table 2. Pharmacological aspects of hispidulin.

No.	Activity	IC_{50} (μM)	% Inhibition (at conc.)	Methodology	Reference
1	Antioxidant	NT	50 (10^{-5}M)	Lipid peroxidation inhibition	(134)
2	Anticancer	20	80* (100 μM) MTS assay (3)		
		20 (at 72 h)	90* (100 μM)	MTT assay	(2)
3	Antiepileptic	1.3	NT	H-flumazenil-BZD binding inhibition	(27)
		8	81 (100 μM)	Maximal GABA response	(89, 135)
		22	80 (100 μM)	Glutamate release inhibition	(57)
4	Anti-Hypnotic	NT	2 (10 nM)	POP inhibition	(91)
			3 (1 μM)		
			43 (100 μM)		
5	Cytotoxic	15.5[a] (Lung Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		7.6[a] (Breast Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		10.4[a] (Cervical Adenocarcinoma)	NT	MTT colorimetric assay	(71)
		5 (Cervical Adenocarcinoma)	NT	MTT colorimetric assay	(50)
		NT	43 (200 μM)	NADH oxidase inhibition	(65)
6	Anti-Influenza	13.9[a]	NT	Influenza H_1N_1 virus neuraminidase inhibition	(52)
7	Antidiabetic	0.49	NT	DPP-IV inhibition	(136)

* Digital values estimated from graphical data presented in relevant articles. [a]μg/mL; NT = not tested; POP = Prolyl oligopeptidase.

Antiinflammatory 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 CCl_4 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 toxicated animals relative to control (non-intoxicated). 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 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 antiosteoclastogenesis drugs are report-

ed 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.

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ANALYSIS

EFFECT OF STORAGE TEMPERATURE ON THE STABILITY OF TOTAL PARENTERAL NUTRITION ADMIXTURES PREPARED FOR INFANTS

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Abstract: Physical, chemical and microbiological stability of total parenteral nutrient (TPN) admixtures was studied as a function of storage time and temperature. Particle size analysis and zeta potential measurements were carried out to evaluate the possible changes in the kinetic stability of the emulsions as a function of storage time and temperature. The concentration changes of the applied additives, those of the ascorbic acid and L-alanyl-L-glutamine, were also determined under different storage conditions. Our results indicate that there were no significant differences in the particle size and zeta potential values of admixtures stored at the three examined temperatures. The best results were obtained in the case of admixtures stored at 30°C temperature. Rapid decomposition of vitamin C was found while the glutamine showed adequate stability as a function of storage time and temperature. According to the results of the physicochemical examinations 10-day storage period of this type of TPN admixtures can be accepted at room temperature. Their storage does not require refrigeration (2-8°C) thus they can be administered without special preheating ensuring better physiological tolerance. Ascorbic acid can be added to the system preceding the administration to the patient because of its rapid decomposition.

Keywords: kinetic stability, all-in-one parenteral nutrition admixture, droplet size distribution, ascorbic acid, L-alanyl-L-glutamine

In the clinical practice, a number of diseases are known that require parenteral nutrition of the patients. Parenteral nutrition should be able to cover the energy needs of the organization and to it should provide the stability of fluids and electrolytes. Accordingly, for parenteral nutrition admixtures contain amino acids, glucose, electrolytes, and fat emulsion in addition to the required vitamins and trace elements. The nutrients can be administered separately, but most often the all-in-one mixtures are applied, taking into account the clinical and economical aspects. However, in contrast to these benefits, a strong limiting factor is the lowering of stability of these multicomponent systems (1). Total Parenteral Nutrition (TPN) admixtures are heterogeneous disperse systems, lipid emulsions, which are thermodynamically unstable (2). Their composition is adjusted to the therapeutic requirements and consequently individualized therapy could be assured to the patients (3). The large number of components and the changing ratio of the additives greatly affect the stability of TPN emulsions (4). Vitamin preparations can protect intravenous lipid emulsions from peroxidation. The administration of multivitamins with the intravenous lipid emulsions provides a practical way to reduce peroxidation of the lipid while limiting vitamin loss (5, 6).

The most critical parameter is the physical stability of systems (2, 7, 8), including the droplet size of the emulsions. If the droplet size exceeds the size of erythrocytes (6-8 μm), embolism can occur, which is of fatal consequences. The droplet size is influenced by several factors; e.g., the added electrolytes by changing the charge of the droplet surface (zeta potential). The droplets due to their surface charge repel each other, thus impeding the coalescence of the droplets (9). In the course of the stability tests phase separation can be observed, but it can be eliminated by simple mechanical influences, although this state is followed by coalescence as

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well (4). The droplet size and droplet size distribution can be usually determined by dynamic light scattering principle operating methods and information can be derived from the charging of the surface by measuring the electrophoretic mobility (zeta potential), that also provides information about the kinetic stability of the system (9, 10).

Chemical degradation is the most important cause of vitamin losses in TPN admixtures. The latter was tracked by the concentration changes of ascorbic acid and L-alanyl-L-glutamine. Vitamins have been mostly quantified in several matrices by HPLC or capillary electrophoresis (11, 12). Few HPLC methods have been developed for simultaneous determination of water- and fat-soluble vitamins (13, 14). The ascorbic acid of parenteral solution was determined by HPLC with UV detection (15). Literature shows that the concentration of ascorbic acid in parenteral solution is out of the acceptable limit (> 90%) (to 82% and 87%) after 48 h at room temperature (25°C). Ascorbic acid was determined after ultrafiltration of the parenteral solution samples by tandem mass spectrometer (ESI-MS/MS) with a minor modification of the literature method (16). The stability assay took 14 days at three temperatures (2-8°C, 25°C, 30°C).

The immune response in critically ill patients is often impaired. Since the glutamine stimulates the transport of nitrogen and reduces the catabolism of the proteins in intestinal or skeletal muscle, therefore glutamine is quickly becoming essential ones due to its increased degradation, thus patients in critical conditions must get exogenous glutamine supplementation.

From the point of practical and safety reasons it could simplify the handling of these systems in the hospital wards without the use of special storage conditions.

Therefore, the primary aim of this study was to investigate the effect of storage temperature on the kinetic stability of the system characterizing by the average particle size, particle size distribution and zeta potential. Another aim of the paper was to examine the degradation of those components sensitive to degradation (ascorbic acid and the glutamine) with monitoring their concentration changes at different temperatures (2-8°C, 25°C, 30°C) for 14 days.

EXPERIMENTAL

Materials

Table 1 summarizes the composition of the prepared TPN admixtures. Ascorbic acid, L-alanyl-L-glutamine, glycylglycine and 3-hydroxy-butyric acid standard were purchased from Sigma-Aldrich (purity > 99.0%).

Table 1. Composition of the TPN admixtures.

Components of the TPN admixture	Packaging [mL]	Quantity (mL)
Infusio glucosi 20% (University Pharmacy, Semmelweis University, Budapest, Hungary)	500	470
Peditrace N inj. (Fresenius Kabi AB, Uppsala, Sweden)	10 × 10	10
Calcimusc inj. (Ca-gluconate 10%, Gedeon Richter Plc., Budapest, Hungary)	10 × 5	20
NaCl 10% inj. (TEVA Pharmaceutical Works Ltd., Debrecen, Hungary)	10 × 10	32
KCl 10% inj. (Pharmamagist Ltd., Budapest, Hungary)	100 × 10	27
Soluvit N inj. (Fresenius Kabi AB, Uppsala, Sweden)	10 × 10	10
Aminoven infant 10% (Fresenius Kabi AB, Uppsala, Sweden)	100	100
Dipeptiven inf. (alanyl-glutamine dipeptide 20%) (Fresenius Kabi Deutschland GmbH)	50	25
Panangin inj. (452 mg potassium aspartate (anhydrous) and 400 mg magnesium aspartate (anhydrous) per 10 mL vial) (Gedeon Richter Plc., Budapest, Hungary)	5 × 10	15
Glucose-1-Phosphate inj. (Fresenius Kabi Austria GmbH, Graz, Austria)	5 × 10	7
Smoflipid 20% inj. (Fresenius Kabi AB, Uppsala, Sweden)	100	100
Vitalipid infant inj. (Fresenius Kabi AB, Uppsala, Sweden)	10 × 10	10

Zeta-potential measurements

Zeta potential measurements were carried out at 25°C using Zetasizer Nano ZS apparatus (Malvern Instruments, UK). An electric field was applied to the dispersion of particles, which would then move with a velocity related to their zeta potential. This velocity was measured using a patented laser interferometric technique called M3-PALS (Phase Analysis Light Scattering). This enables the calculation of electrophoretic mobility, and from this the zeta potential for the accurate measurement using the Smoluchowsky formula and expressed in mV. Measurements were carried out at 25 ± 1°C with the freshly prepared emulsions and also, with the samples stored for 0 to 14 days on 3 different temperatures. Particle size range for zeta potential measurement is from 5 nm to 10 μm.

The evaluation of zeta potential measurements indicates the instability of these systems with the values smaller than ±30 mV. The results showed decreasing tendency especially in samples stored at low temperature.

Particle size measurements

Mean droplet size (MDS), size distribution and polydispersity of the emulsion droplets were measured at 25°C using Zetasizer Nano ZS apparatus (Malvern Instruments, UK).

Dynamic Light Scattering (DLS) is used to measure particle diameter. This technique measures the diffusion of particles moving under Brownian motion, and converts this to size and a size distribution using the Stokes-Einstein relationship. Non-Invasive Back Scatter technology (NIBS) is incorporated in the instrument to give the highest sensitivity simultaneously with the highest dynamic size and concentration range. It includes 2 angle size measurements for the enhanced detection of aggregates, measurement of small or dilute samples, or at high concentration. In addition, the optics is not in contact with the sample and hence the detection optics are said to be non-invasive. The measurement position within the cuvette of the Nano ZS is automatically set to accommodate the requirements of high sensitivity or high concentration. This position is changed by moving the focusing lens.

Particle size range for size measurement is from 0.6 nm to 6 μm.

Statistical evaluation

Average particle size and zeta-potential values of mixtures at different temperatures and storage intervals were compared using the two-sample t-test assuming equal variances. In this case, the comparison was made between infusions stored at different temperatures.

The statistics were calculated using Microsoft Excel 2003.

Determination of the ascorbic acid concentration

The measurement was run by the internal standard method with 3-hydroxy-butyric acid internal standard. Ascorbic acid was diluted in deionized water to obtain solutions at appropriate concentrations for implementation. They were freshly prepared before use. The samples were ultrafiltrated with 10 kDa filter (Millipore) at 14000 rpm before measuring them. The ESI-MS/MS measurements were accomplished with API 4000 QTRAP MS/MS mass spectrometer equipped with Perkin Elmer 200 LC. The ESI-MS/MS system was used in negative ion mode, the quantification of the ascorbic acid ($M(M-H^+)$ = 175) and the internal standard ($M(M-H^+)$ = 103) based on transitions of *m*/*z* 175→115, *m*/*z* 175→87 and *m*/*z* 103→77. For the optimum MS/MS performance the measuring parameters were: ion spray voltage: -4500 V, ion source temperature: 200°C, declustering potential: -60 V, entrance potential: -10 V, collision energy -24 V. Twenty mL sample was injected in infusion mode. The eluent was water : acetonitrile mixture (80 : 20, v/v) with flow rate of 200 μL/min.

Examination of the stability of L-alanyl-L-glutamine

The stability assay took 14 days at three temperatures (2-8°C, 25°C, 30°C). The measurement was acc. to the internal standard method with glycyl-glycine internal standard. The L-alanyl-L-glutamine was diluted in deionized water to obtain solutions at appropriate concentrations for implementation. The ESI TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with Jasco X-LC binary pump and Jasco X-LC autosampler was used. The ESI-MS/MS system was used in positive ion mode, the quantification of the L-alanyl-L-glutamine ($M(M+H^+)$ = 218) and the internal standard ($M(M+H^+)$ = 133) was based on transitions of *m*/*z* 218→84, *m*/*z* 218→130, *m*/*z* 218→147 for the first compound and *m*/*z* 133→76, *m*/*z* 133→87 for the second. The MS parameters were: spray voltage: 4000 V, sheat gas pressure: 35 psi, aux gas pressure: 5 psi, capillary temperature: 300°C, collision pressure: 1.0 mTorr, scan time: 0.3 s, tube lens offset: 70 V.

Microbiological examinations

The parenteral formula was studied at three different temperatures (2-8°C, 25°C, 30°C) for 14

consecutive days. Aerobic bacterial and mycological cultures were prepared according to the pharmacopoeia monographs.

The samples were treated and evaluated according to the rules of microbiological sample processes. Ten mL of tested TPN samples were inoculated onto Columbia agar containing 5% sheep blood, onto chocolate agar containing polyviteX and onto fungal Sabouraud medium (BioMérieux plates). They were incubated at 37°C for 24 h, then stored at room temperature for another 24 h. Reading and evaluation of the discs were carried out after 24 and 48 h. One mL of the TPN solution was inoculated onto medium, which contains hemin and vitamin K3 thioglycolate, and incubated at 37°C for 24 h. The enriched sample was processed and evaluated in the same manner of the direct blanking.

RESULTS

The most important condition of the nutrition is that the foods should be digested and the nutrients in the digestive system can convert into small molecules. A suitable zeta potential (negative electrical charge) is required, i.e., the nutrients

Table 2. The results of the particle size analysis at different storage temperatures.

Storage time /days	Storage temperature		
	2-8°C	25°C	30°C
	Particle sizes (nm) and relative intensity values		
0	694.9	694.9	694.9
3	497.1	499.2	517.9
4	609.4 – 95.3% 2537 – 4.7%	698.2 – 95.3% 2532 – 4.7%	508.7
5	583.2 – 94.5% 4213 – 5.5%	588.1 – 97.3% 4012 – 2.7%	543.1
6	361.4 – 93.4% 4513 – 6.6%	605.4 – 94.9% 4773 – 5.1%	412.6 – 96.5% 4044 – 3.5%
7	484.6 – 97.6% 5048 – 2.4%	634.3 – 98.4% 4981 – 1.6%	547.5 – 95.2% 5012 – 4.8%
10	497.2 – 97.1% 5205 – 2.9%	698.0 – 97.9% 4997 -2.1%	633.6 – 95.6% 5071 – 4.4%
11	496.0 – 95% 5195 – 5%	655.5 – 96% 5022 – 4%	606.7 – 94.9% 5077 – 5.1%
12	502.5 – 94.5% 5198 – 5.5%	611.3 – 95.6% 5028 – 4.4%	598.8 – 94.2% 5089 – 5.8%
13	499 – 95.2% 5144 – 4.8%	627.2 – 94.8% 5042 – 5.2%	601.3 – 94.2% 5097 – 5.8%
14	400.2 – 94.8% 5187 – 5.2%	624.7 – 93.8% 5087 – 6.2%	596.4 – 93.6% 5100 – 6.4%

Table 3. Results of the two-sample t-test of admixtures stored at different temperatures.

Zeta-potential measurements		
Between 2-8°C and 25°C	p = 0.00071	0.071%
Between 2-8°C and 30°C	p = 0.0017	0.17%
Particle size measurements		
Between 2-8°C and 25°C	p = 0.00071	0.071%
Between 2-8°°C and 30°C	p = 0.0462	4.62%

p refers to the comparison of the zeta-potential and average particle size values with the corresponding values at 2-8°C.

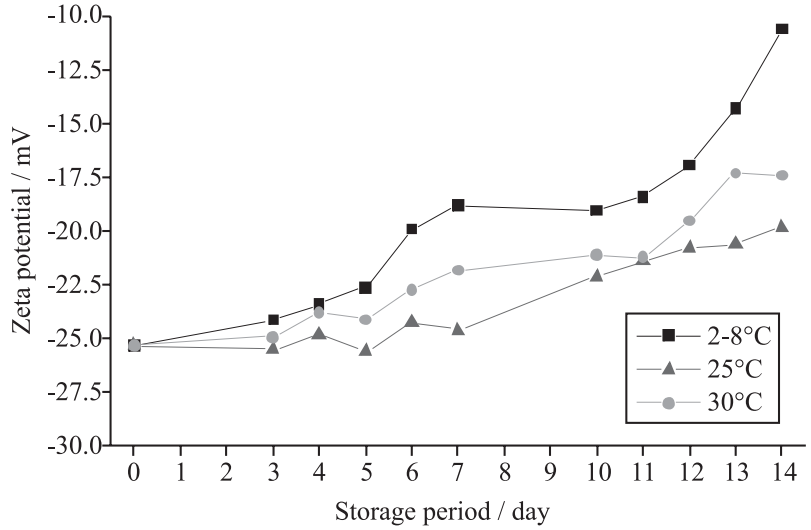

Figure 1. Zeta potential values measured at different storage temperatures as a function of storage time

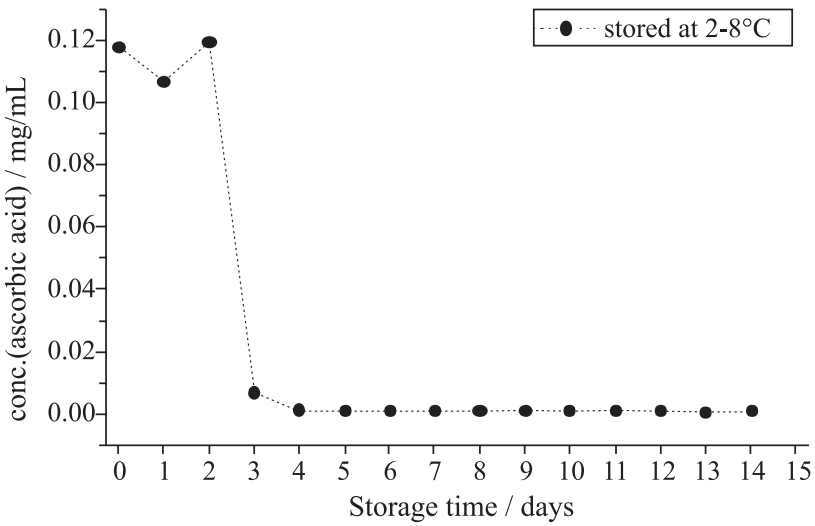

Figure 2. Changes in the concentration of ascorbic acid in the samples stored at 2-8ºC

have to get easily into the cells and to leave from there. As long as the negatively charged nutrients and positively charged intracellular fluid are maintained in equilibrium, the cell metabolism is proper and the way you feel is good. Figure 1 illustrates that the negative zeta potential remained under each storage temperature in the whole 14-day-storage period.

If a patient is unable to be nourished, the parenteral infusion is lifesaver, but efforts should be made to induce fewer side effects with it. It is particularly important that the fat emulsion must have smaller particle size. Under each storage conditions the particle size of the admixtures not even come close to 10 microns during 14 days long study. Table 2 summarizes the particle size distributions of samples stored under different temperatures. The larger particles, which were present in average in 4.6%, did not exceed an average size of 5 microns long red blood cells.

DISCUSSION AND CONCLUSION

There were no significant differences in the average particle size and zeta potential values of admixtures depending on the storage temperatures (Table 3). After examination of the samples, the storage time exceeded even 14 days at all three

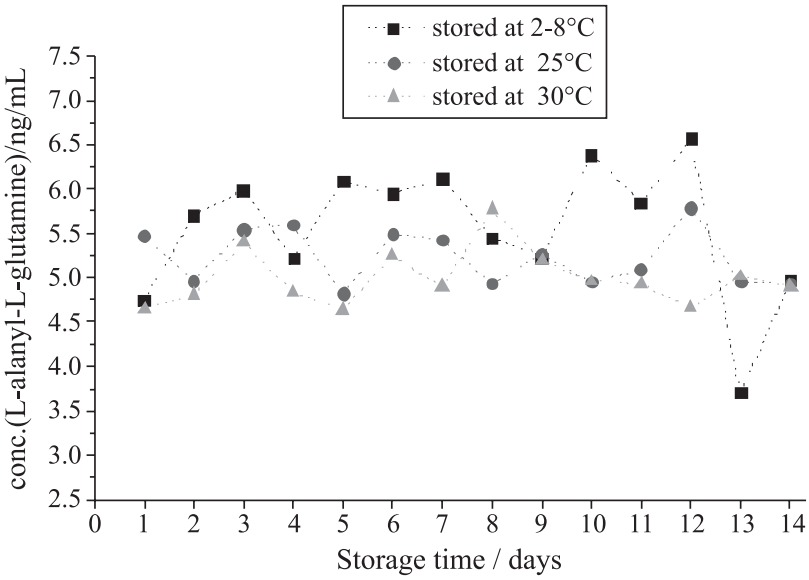

Figure 3. Changes in the concentration of L-alanyl-L-glutamine at different temperatures as a function of storage time

temperatures. Moreover, the results show that the values were the best at 30°C; the 4044 nm particle appeared in 3.5% only on the 6th day. However, it was not possible to detect ascorbic acid at 25°C and 30°C after 24 h. Samples, which were stored at 2-8°C, contained ascorbic acid after 48 h, but after 3 days, the ascorbic acid became immeasurable, hence completely decomposed in each sample (Fig. 2). In contrast to the ascorbic acid, L-alanyl-L-glutamine can be stored for 14 days without decomposition at each of the examined temperature. Figure 3 confirms that the concentration changes of L-alanyl-L-glutamine remained within the acceptable limits. The relative standard deviations (% RSD) are under 10% (6.3 - 9.5%). Each sample remained sterile within the whole storage interval.

According to the results of the physicochemical examinations, 10-day storage period of this type of TPN admixtures at room temperature can be accepted. Their storage does not require refrigeration (2-8°C), thus they can be administered without special preheating ensuring better physiological tolerance. Because of the rapid decomposition of vitamin C, the water-soluble Soluvit multivitamin has to be added into the admixture in every day, while the glutamine can be mixed with the amino acid infusion because of its adequate stability.

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SELECTED ASPECTS OF TERAHERTZ SPECTROSCOPY IN PHARMACEUTICAL SCIENCES

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Abstract: THz-TDS techniques are applied to investigate selected pharmaceutical samples. Investigations were performed on selected pharmaceutical samples with active pharmaceutical ingredients (API) - famotidine, ranitidine, fenofibrate, lovastatin, simvastatin, aspirin, ketoconazole, acyclovir (hydrated and non-hydrated), on excipients - lactose, glucose (hydrated and non-hydrated), Pluronic 127, and on mixtures of selected compounds. Pseudo-polymorphism effects are considered as well. Examples of the terahertz imaging technique are also given. APIs and excipients can be easily recognized in the terahertz band by their specific "fingerprints" as individual components and in mixtures. The hydration process as a variety of polymorphism can also be easily monitored using the THz technique. Moreover, terahertz light can be useful for the penetration of tablets, giving clear pictures of possible defects in tablet coatings.

Keywords: terahertz technique, pharmaceutical sciences, time-domain spectroscopy, pseudo-polymorphism, hydration, coating, imaging, THz, far-infrared absorption

Nowadays, terahertz technology is definitely not a "dead land", as it was long ago described (1). Terahertz techniques are setting new trends in research methodology used in many domains of biomedicine, including the pharmaceutical sciences (2-7). Waves from the band of the electromagnetic spectrum called THz radiation (10-333 cm^{-1} = 0.3-10 THz) are being successfully harnessed for investigations on pharmaceutical media by utilizing significant spectral information. Terahertz spectroscopy contains information about crystal lattice vibrations that are associated directly with the structure of the crystal. In contrast, so called vibrational spectroscopy - the domain of the MIR and NIR spectrum - focuses its interest on the structure and dynamics of molecules in gases, liquids and at interfaces. In other words, it deals with vibrational motions including chemical reaction dynamics. Spectra extracted from a THz region are very sensitive to structural properties of media including pharmaceutical ingredients (8). In precisely this spectral region "heavy" molecules, which are predominantly used in the pharmaceutical domain, leave so called "fingerprints" - specific distributions of absorption lines, or in other words, spectral details (the "fingerprints"). It spans approximately between 10 and 200 cm^{-1}. The bandwidth is rather fluid and is still being modified by results appearing in scientific journals, even from 8 to 220 cm^{-1} (6, 7, 9-12). The THz technique allows the monitoring of pharmaceutical products throughout the development process, recognizing the specific spectra of the drug components. Identification of the polymorphs or hydrates in drug forms is becoming more and more complicated with

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the development of contemporary pharmacology and drug form technology (13). There are also many methods of the analysis: dissolution testing, thermoanalytical methods, calorimetric analysis, microscopic methods (14, 15), X-ray diffraction (XRD) (13), differential scanning calorimetry (DSC) and FTIR spectroscopy (16). Apart from spectroscopic methods (IR, FTIR, Raman), THz spectroscopy can be used for this purposes, especially to investigate solid dosage forms to demonstrate e.g., pseudopolymorphism (hydration effects) (17). Terahertz waves can substitute for dangerous and not "comfortable" X-radiation for investigations of cross sections of the drugs without destruction of the sample (13).

MEASUREMENT SETUP

In this paper we utilize pulsed THz generators used in the arrangement of terahertz time domain spectroscopy (THz-TDS) (18) to measure the spectral properties of investigated media. In order to verify our results we used typical substances of known data. To illustrate the problem, we present the results of measurements of the following: APIs such as famotidine, ranitidine, fenofibrate, lovastatin, simvastatin, aspirin, ketoconazole, acyclovir hydrated, acyclovir non-hydrated; excipients such as lactose, glucose hydrated, glucose non-hydrated, Pluronic 127; mixtures such as fenofibrate with aspirin = 44/56, lovastatin with aspirin = 27.8/72.2, lovastatin with aspirin = 83.6/16.4, pluronic with ketoconazole = 50/50.

Additionally, an example of terahertz imaging was performed on a ketonal tablet with coating in THz-TDS reflective arrangement.

Terahertz TDS spectrometer arrangement

A standard terahertz time domain spectroscopy measurement system is shown in Figure 1. The system contains a femtosecond laser which drives the emitter Tx and receiver Rx antennas. The pellet is placed in the focus of the THz beam collimated by parabolic off-axis mirrors PM. The beam of the laser is being split with a cubic beam splitter BS into beams "1" and "3" and next, focused with microscopy lenses on Tx and Rx antennas. The coherent homodyne detection method is used for the investigations (1). The THz beam path "2" is purged with dry air to avoid absorption by water vapor. The samples are used in polycrystalline form and mixed with polyethylene (PE) powder. PE powder is used because it is nearly transparent in the terahertz region (19).

THEORY

When a sample is inserted in the arm - see Figure 2 - the test material, the Rx antenna measures the signal E_{med}^{Thz} with the presence of some medium in the arm. The result is a convolution of two functions - terahertz E_{ref}^{Thz} and optical E_{prob} signals - without the sample, and terahertz E_{med}^{Thz} and optical E_{prob} signals - with the sample. The idea of the measurements and calculations is illustrated in Figure 2. The method is called a coherent homodyne detection of the signal. In fact, it is a homodyne setup, where the same optical beam (an exciting beam) creates the terahertz wave in phase with the optical beam, and simultaneously the same optical beam is used as a probing one.

As is known, the conversion of such signals from time domain to frequency domain makes the

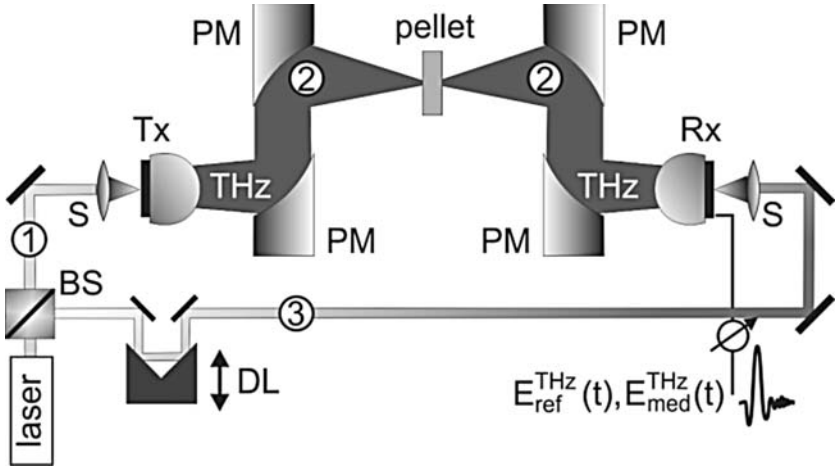

Figure 1. Schematic illustration of the THz-TDS spectrometer. 1 + 2 – measurement arm of the spectrometer, 3 – probing arm, Tx – THz transmitter, Rx – THz receiver, BS – cubic beam-splitter, DL – optical delay-line, PM – parabolic off-axis mirrors, S – microscopy lenses

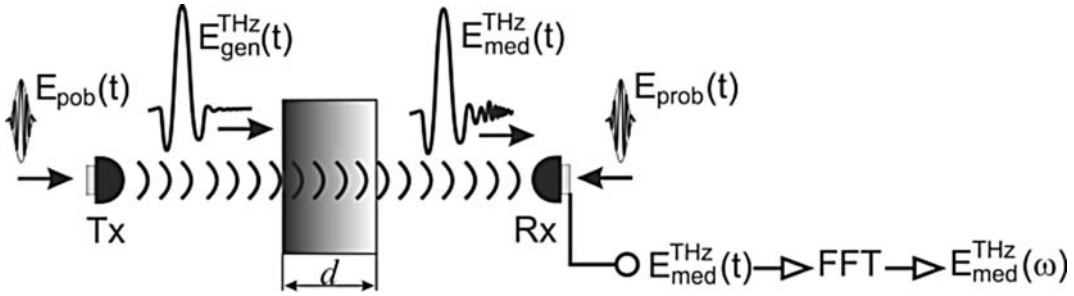

Figure 2. Coherent homodyne detection – the measuring and calculations of the signal with the sample, where d – thickness of the sample. FFT – Fast Fourier Transform operation on the measured signal

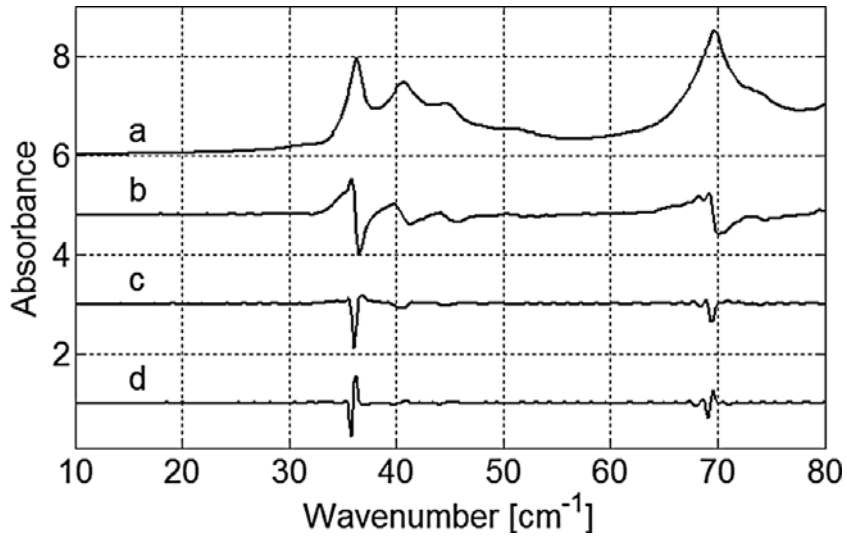

Figure 3. Famotidine spectrum (a), first (b), second (c), and third (d) derivatives of the spectrum

calculations easier. The signals are converted from time domain to frequency domain using the Fourier transform. Then, the convolution function can be reduced to their ordinary multiplication.

The spectrum of the investigated sample is expressed as an absorption coefficient *versus* frequency :

$$\alpha(\omega): -\frac{2}{d} \cdot \ln \frac{E_{med}^{Thz}(\omega)}{E_{ref}^{Thz}(\omega)} \qquad (1)$$

where: d - difference between sample and reference tablet thickness; E_{ref}^{Thz} and E_{med}^{Thz} amplitudes of the reference signal and the signal with the sample medium, respectively.

Improving spectrum visual analysis

The investigation methodology is explained on the example of the famotidine. For better identification of the absorption lines, first, second and third derivative of the spectrum can be used - see Figure 3. All of them have some advantages. Odd derivatives make sense for correct estimation of the central frequency of the absorption lines, because it is possible to recognize a clear transition of the characteristic through the zero level of the measured signal, which indicates the position of the absorption peak. The third derivative method has another advantage - it eliminates the background. On the other hand, the second derivative method is the most comfortable, and was used in our experiment.

Measurements and materials

The investigations are divided into four measurement groups:

- terahertz recognition of drugs commonly used in pharmaceutics - API,
- typical pharmaceutical excipients in the THz range,

- recognition of compounds in mixtures,
- visualization of pseudo-polymorphism or, in other words, hydration effects.
- additionally to spectroscopy we show results of the tablet coating imaging.

Terahertz recognition of drugs

We investigated APIs commonly used in pharmacy. In our studies APIs from different pharmacological groups such as histamine H2 receptor blockers and hypolipidemic drugs of the statin and fibrate groups were analyzed.

- Famotidine (3-[({2-[(diaminomethylidene)amino] -1,3-thiazol-4-yl}methyl)sulfanyl]-N'-sulfamoylpropanimidamide) is a histamine H2 receptor antagonist. It is widely used for the treatment of stomach ulcers and gastroesophageal reflux disease. Famotidine has been intensively investigated in the terahertz region (20). Famotidine was provided as a gift by Polfa S.A. (Kutno, Poland).
- Ranitidine (N-(2-[(5-[(dimethylamino)methyl] furan-2-yl)methylthio]ethyl)-N'-methyl-2-nitroethene-1,1-diamine) is, like famotidine, a histamine H2-receptor antagonist that inhibits stomach acid production. It is commonly used in the treatment of peptic ulcers and gastroesophageal reflux disease. Ranitidine was purchased from Farchemia S. r. l. (Bergamo, Italy).
- Fenofibrate (propan-2-yl 2-{4-[(4-chlorophenyl) carbonyl]phenoxy}-2-methylpropanoate) helps reduce cholesterol, triglicerydes and both low-density lipoprotein (LDL) and very low density lipoprotein (VLDL) levels in the blood. Fenofibrate was purchased from Sigma-Aldrich (Steinheim, Germany).
- Lovastatin and simvastatin are members of a pharmaceutical class of drugs called HMG-CoA reductase inhibitors, which have pleiotropic effects manifested by protective effects on vascular endothelium, plaque stabilization, anti-inflammatory effect. Lovastatin ((1S,3R,7S,8S,8aR)-8-{2-[(2R,4R)-4-hydroxy-6-oxooxan-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl (2S)-2-methylbutanoate), belongs to class of statins, is used like fenofibrate to reduce cholesterol level in the blood. Lovastatin was obtained from Polpharma S.A. (Starogard Gdański, Poland).
- Simvastatin ((1S,3R,7S,8S,8aR)-8-{2-[(2R,4R)-4-hydroxy-6-oxotetrahydro-2H-pyran-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8a-hexahydronaphthalen-1-yl 2,2-dimethylbutanoate) is the methylated form of lovastatin and has a similar clinical application. Simvastatin was obtained from Polpharma S.A. (Starogard Gdański, Poland).
- Acetylsalicylic acid (2-acetyloxybenzoic acid), also known as aspirin, has been used as an analgesic, antipyretic and anti-inflammatory nonsteroidal drug. A low-dose of acetylsalicylic acid has an inhibiting effect on platelet aggregation

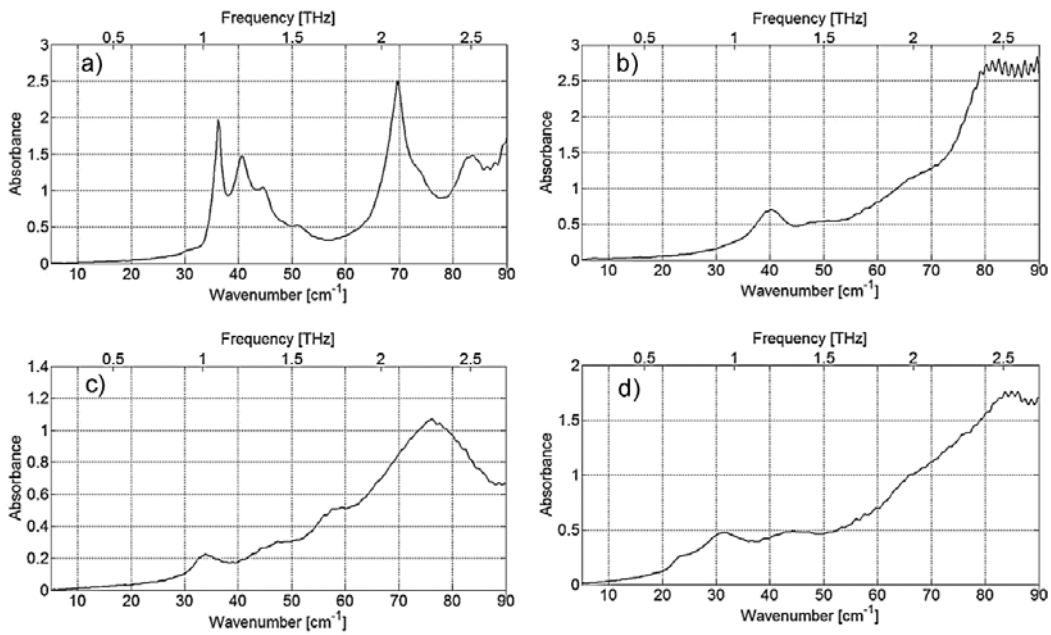

Figure 4. a) spectrum of famotidine in the terahertz range, b) terahertz spectrum of ranitidine, c) THz spectrum of fenofibrate, d) THz spectrum of lovastatin

and is applied to prevent atherosclerotic cardiovascular disease. Aspirin has been intensively investigated in the terahertz region (21). Acetylsalicylic acid was purchased from Sigma-Aldrich (Steinheim, Germany).

- Ketoconazole (1-[4-(4-{[(2R,4S)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazin-1-yl]ethan-1-one) is one of the azole-based antifungal drugs used to treat infections caused by a fungus or yeast. Ketoconazole was acquired from Hasco-Lek S.A. (Wrocław, Poland).
- Acyclovir (2-amino-1,9-dihydro-9-[(2-hydroxyethoxy)methyl]-6H-purin-6-one; chemical name - acycloguanosine) is one of the most commonly used antiviral drugs - primarily used for the treatment of herpes simplex virus infections. Acyclovir was provided as a gift by Sanitas Group (Jelenia Góra, Poland).

Pharmaceutical excipients in the THz range

An excipient is generally a pharmacologically inactive substance used as a carrier for the APIs. The US Pharmacopeia-National Formulary (USP-NF) categorizes excipients as binders, disintegrants, diluents, lubricants, glidants, emulsifying-solubilizing agents, sweetening agents, coating agents, antimicrobial preservatives, and so forth (22). In pharmaceutical technology these substances are widely used in direct compression tableting applications, and as a tablet and capsule filler and binder. Fillers are inert ingredients that can significantly affect the chemical and physical properties of the final tablet thus affecting the biopharmaceutical profile (23). Binders, in turn, are added to tablet formulations to add cohesiveness to powders thereby providing the necessary bonding to form granules which, under compaction, form a compact mass as tablet. In other words, binders are essential to achieve the "hardness" of the tablet (24).

- Lactose in the drug form technology is a basic filler used in the manufacture of granules and tablets. This substance is a hydrophilizing agent used in the process of direct compression. Lactose monohydrate was purchased from Sigma-Aldrich (Steinheim, Germany). Lactose has been investigated in the FIR region (25).
- Glucose is the filler substance in granules, chewable tablets and vaginal tablets. It is also used as a binder in the wet granulation process. The granules obtained are difficult to dry due to the hygroscopic properties of glucose. Tablets with the addition of glucose harden during storage what extends the time of their disintegration. Glucose anhydrous pure p. a. was purchased from Chempur (Piekary Śląskie, Poland). Glucose has been intensively investigated with DSC methods (26).

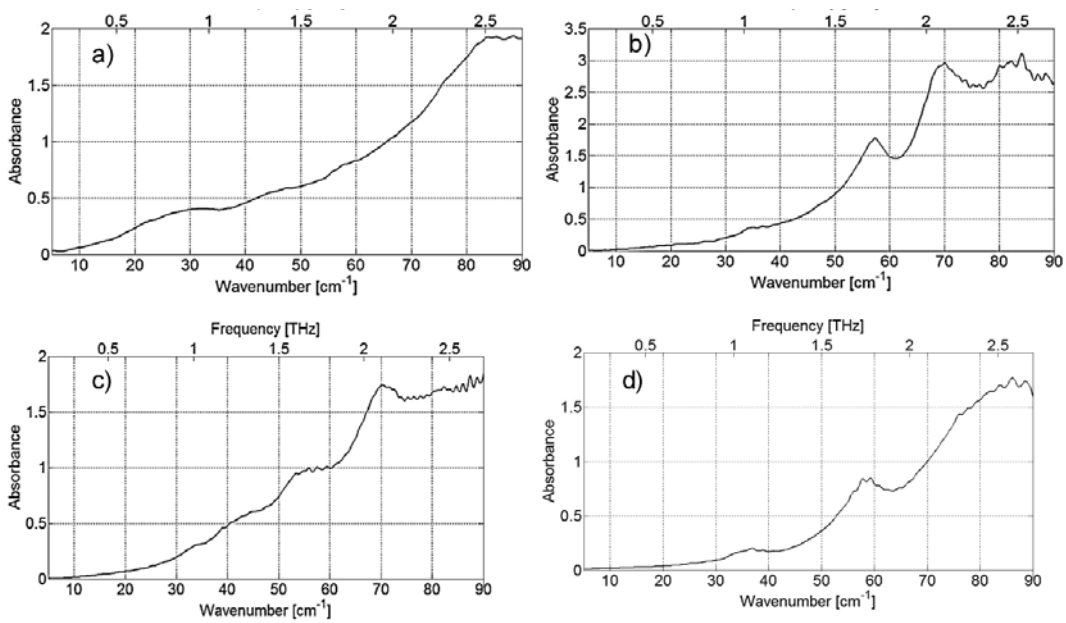

Figure 5. a) THz spectrum of simvastatin, b) THz spectrum of acetylsalicylic acid (aspirin), c) ketoconazole terahertz spectrum, d) acyclovir hydrated spectrum

- Pluronic F127 is a polymer of polyoxyethylene (PEO) and polyoxypropylene (PPO) with two 96-unit hydrophilic PEO chains surrounding one 69-unit hydrophobic PPO chain. Pluronic is exploited in pharmaceutical formulations as an emulsifier, wetting agent and solubilizer for poor water soluble drugs. The use of this surfactant in formulations containing a polymeric carrier may help prevent precipitation and/or protect a fine crystalline precipitate from agglomeration into much larger hydrophobic particles. The inclusion of pluronic as a carrier, was shown to be effective in enhanced in vivo substance bioavailability (27). Pluronic® F-127 was purchased from Sigma-Aldrich (Steinheim, Germany).

RESULTS

The results of investigations are divided into three groups:

- terahertz recognition of APIs commonly used in pharmaceutics,
- typical pharmaceutical excipients in the THz range,

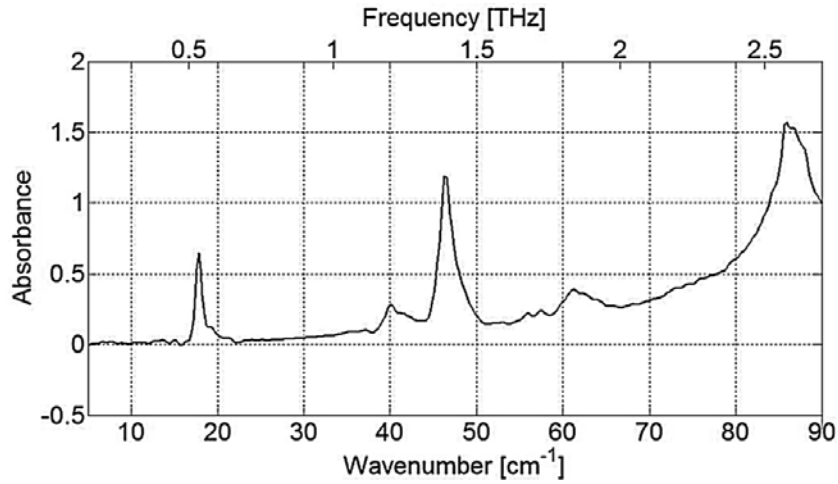

Figure 6. Terahertz spectrum of lactose monohydrate

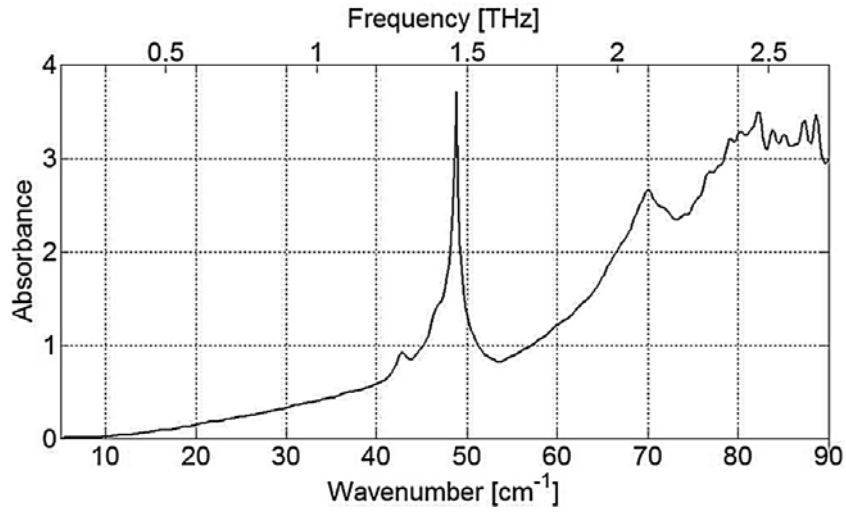

Figure 7. Terahertz spectrum of glucose (non-hydrated)

Table 1. Collected results of the drug spectral measurements in terahertz region, and comparison with the literature. (A) and (B) – polymorphic forms of famotidine (20, 29).

Compound	Absorption line (cm^{-1})
Drugs	
Famotidine	36.3, 40.5, 44.5, 51.0, 69.7, 74.0, 83.6
(29) experimental	30.0 (A), 32.3 (A), 35.0 (B), 35.3 (B), 39.6 (B), 40.0 (B), 44.6 (B), 51.1 (B), 54.0 (A), 55.0 (A), 62.2 (A), 70.7 (A), 80.8 (A), 85.2 (A)
(29) calculated	32.21, 37.89, 44.85, 55.33, 59.83, 72.74, 83.43, 92.1, 97.17
(20)	30.0 (A), 40.0 (B), 46.6 (B), 53.3 (A)
(20)	36.0, 40.0, 43.0, 53.0, 68.0, 73.0, 82.0, 93.0
Ranitidine	40.0, 50.0, 66.8
(30)	Form 1: 31.6, 41.9, 50.9, 59.0, 67.9, 73.3, 84.9, 91.9 Form 2: 37.6, 46.6, 61.6, 75.3, 79.6, 91.9
Fenofibrate	33.9, 46.5, 57.5, 76.0
Lovastatin	22.4, 32.3, 47.0, 57.9, 85.5
Simvastatin	22.5, 29.9, 47.0, 57.8, 76.0, 85.5
Aspirin	34.5, 36.7, 47.0, 57.4, 69.5, 81.9
(21)	37.0, 62.3, 72.3, 78.3, 82.9, 95.6, 111.2, 122.9
(21)	55.9, 71.3 (31) 18, 38.5, 53.5, 60.0, 62.5, 72.5, 76.0, 78.5, 82.5, 88.0, 93.0
(32)	10, 18, 34, 37, 46, 59, 66, 73, 81, 91, 100
Ketoconazole	33.7, 45.0, 55.5, 70.2
Acyclovir hydrated	36.5, 58.5, 77.5
Acyclovir non-hydrated	49.0, 70.4

- tablet coating imaging in terahertz light.

The measurements were performed in the 0.06 - 3 THz (approx.. 2 cm^{-1} - 90 cm^{-1}). The samples were mixed with polyethylene (PE) powder which is transparent to terahertz radiation. Mixtures consisted of 360 mg of PE powder and 40 mg of medium. As reference tablet was used 360 mg PE (HDPE) only (28). Medium was carefully mixed and then pressed into pellets with a Stempel Specac hydraulic press equipped with a 13-mm stainless steel die under the 2 ton pressure for 2 min.

Active pharmaceutical ingredients

The spectrum of famotidine is shown in Figure 4a. Seven absorption lines are indicated: 36.3, 40.5, 44.5, 51.0, 69.7, 74.0 and 83.6 cm^{-1}.

The spectrum of ranitidine in the terahertz region is shown in Figure 4b. Three absorption lines are indicated: 40.0, 50.0 and 66.8 cm^{-1}. The terahertz spectrum of fenofibrate is shown in Figure 4c. Four absorption lines are specified: 33.9, 46.5, 57.5, and 76.0 cm^{-1}. The THz spectrum of lovastatin is shown in Figure 4d. Five lines are visible: 22.4, 32.3, 47.0, 57.9 and 85.5 cm^{-1}.

The THz spectrum of simvastatin is shown in Figure 5a. Six spectral details may be specified: 22.5, 29.9, 47.0 and 57.8, 76.0, and 85.5 cm^{-1}.

The spectrum of aspirin is shown in Figure 5b. Six absorption lines are indicated: 34.5, 36.7, 47.0, 57.4, 69.5, and 81.9 cm^{-1}. The terahertz spectrum for ketoconazole (Figure 5c) is specified for: 33.7, 45.0, 55.5, and 70.2 cm^{-1}. Figure 5d shows the spectrum obtained for acyclovir in non-hydrated form. Two absorption lines are identified: 36.5 and 58.5 cm^{-1}.

Excipients

For the investigations, three known, and typical pharmaceutical excipients - lactose, glucose, and Pluronic F127 were selected. The terahertz spectrum of lactose is shown in Figure 6. Five lines are visible: 17.8, 40.0, 46.5, 61.5 and 86.5 cm^{-1}. The terahertz spectrum of glucose is shown in Figures 7 and 15.

The terahertz spectrum of glucose is shown in Figure 7. Four visible lines are identified for non-hydrated glucose: 42.7, 48.8, 70.0, and 84.7 cm^{-1}.

The terahertz spectrum of Pluronic F127 is shown in Figure 8. Pluronic has been intensively investigated in the terahertz region (7, 12, 21).

It may be observed that, as in the case of APIs, it is easy to recognize the spectra of lactose and glucose. Both show easily observable absorption peaks. The picture is quite different in the case of Pluronic F127. Only one very weak absorption line can be observed at 49.9 cm^{-1} (see Table 2).

DISCUSSION AND COMPARISION WITH THE LITERATURE

The results of spectroscopy measurements of APIs, excipients and their mixtures are discussed in this section. Processes of dehydration effects of glucose and acyclovir are discussed as well. In addition problems of drug homogeneity are also discussed based on the results of terahertz imaging of the tablets.

APIs

The results confirm the possibilities of the terahertz technique in spectroscopy of active pharmaceutical ingredients. Observable absorption peaks in the terahertz region for the investigated ingredients (famotidine, ranitidine, fenofibrate, lovastatin, simvastatin, aspirin, ketoconazole, and acyclovir) give characteristic spectra which are easy to recognize. The collection of absorption lines given in Table 1 creates "fingerprints" which can be easily assigned to suitable ingredients.

As can be seen in Figure 4a, famotidine shows an easily recognized spectrum ("fingerprint") with three well-developed absorption lines at 36.3, 40.5, and 69.7 cm^{-1}, and four weak lines at 44.5, 51.0, 74.0, and 83.6 cm^{-1}. The lines 44.5 and 74.0 cm^{-1} are set on the slope of the characteristic, and this is why estimation of their strength can be confusing. The table contains the results of other authors as well. All lines which are observable in our experiment remain in good agreement with experimentally obtained and calculated lines in (20, 29). Other authors indicated also 30.0, 32.3, 54.0, 62.0, 80.8, 85.2 cm^{-1} and calculated 92.0, and 97.0 cm^{-1} (29); 46.6, 53.3, cm^{-1} (20); 53.0, 93.0 cm^{-1} (33). The lines given in (20, 29) are obtained from two polymorphic forms: (A) - by recrystallization with hot water, and (B) - by recrystallization with hot methanol aqueous solution.

Figure 4b shows the spectrum of ranitidine. We indicated a strong line at 40.0 cm^{-1} and weak lines at 50.0 and 66.9 cm^{-1}. All three lines remain in acceptable agreement with the results of other authors (30). In one paper (30) two polymorphic forms are investigated: form 1 - obtained by crystallization from ethanolic solution after addition of ethyl acetate, and form 2 - obtained from a solution of isopropanol-HCl. The authors also found other lines at 31.6,

Table 2. Collected results of the excipients spectral measurements in terahertz region, and comparison with the literature.

Compound	Absorption line (cm^{-1})
Excipients	
Lactose	17.8, 40.0, 46.5, 61.5, 86.5
(33)	18.0, 40.0, 46.0, 61.0
(35) non-hydrated	30.0, 36.0, 47.0, 54.0, 62.0, 76.0, 87.0, 94.0, 104.0
(35) hydrated	18.0, 40.0, 46.0, 60.0, 85.0, 96.0, 108.0
(34)	18.0, 46.0
Glucose hydrated	61.3, 66.5, 81.9
Glucose non-hydrated	42.7, 48.8, 70.0, 84.7
(36) D anhydrous	43.0, 48.0, 69.9, 86.6
(36) D hydrated	60.6, 65.9, 81.9
(37)	42.3, 47.3, 59.3, 68.3, 83.6, 87.9, 96.9, 110.5, 115.9, 124.5
(38) D-glucose	42.0, 48.3, 69.9
(38) L-glucose	48.3, 70.6
Pluronic	127 49.9

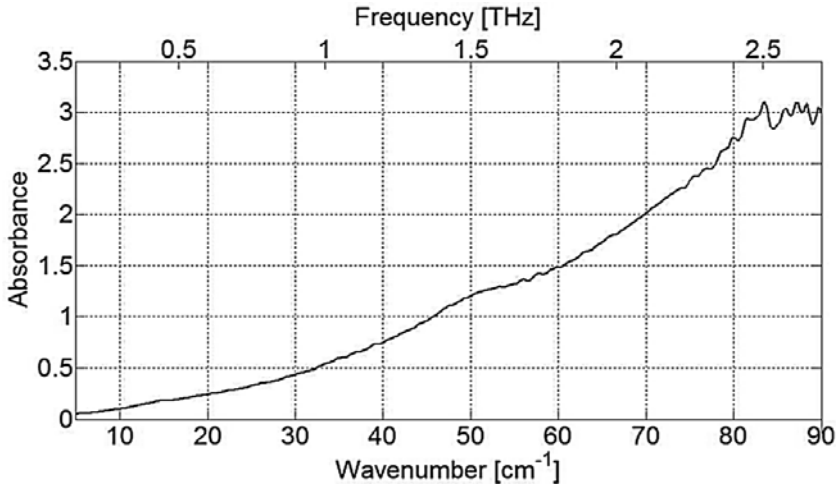

Figure 8. Terahertz spectrum of Pluronic F127

59.0, 73.3, 84.9, 91.9 for form 1, and 37.6, 46.6, 61.6, 75.3, 79.6, 91.9 for form 2. It is very likely that the higher number of lines is a result of the higher amount of the ingredient in the PE tablet (25%) comparing to our experiment (5%).

Fenofibrate (Fig. 4c), lovastatin (Fig. 4d), simvastatin (Fig. 5a), and ketoconazole (Fig. 5c) can be characterized by easily recognized spectra, but they do not show significant absorption lines. The lines for these compounds are collected in Table 1. There are no results of other authors for these investigated molecules.

The aspirin spectrum is presented in Figure 5b. Our experiment reveals five absorption lines - two strong at 57.4 and 69.5 cm^{-1}, and three weak at 34.5, 36.7, 47.0 cm^{-1}. Our spectrum incompletely overlaps with the experimental data of other authors. In one paper (21) aspirin was investigated with two methods - as a tablet and in a waveguide. One of them, at 71.3 cm^{-1}, is in good agreement with our result at 69.5 cm^{-1}, while using the waveguide the authors indicated six lines at 37.0, 62.3, 72.3, 78.3, 82.9, 95.6, 111.2, 122.9 cm^{-1}, where one of them, at 37 cm^{-1} is in a good agreement with our result at 36.7 cm^{-1}. But it is necessary to emphasize that the experiment in this paper (21) was performed at a temperature of 77 K.

The results given in another paper (31) we roughly determined from Figure 4 cited therein. Our results for some selected lines at 36.7, 57.4, and 69.5 cm^{-1} can be approximated to the lines given in this paper (31). The difference may be due to different conditions of both experiments - our experiments were performed at room temperature while the above-mentioned was at 10 K. More spectral features are usually visible at cryogenic temperatures. As we have shown, our results are in very good agreement with theoretical simulations performed in paper (32).

The discussion about acyclovir spectra for both hydrated and non-hydrated samples is to be found below. Figure 5d shows a spectrum for the hydrated form only. As is seen, only two spectral features are visible at 36.5 and 58.5 cm^{-1}.

Excipients

The spectral features of excipients (lactose hydrated, glucose hydrated and non-hydrated, Pluronic 127) are collected in Table 2. Figure 6 shows our results for lactose monohydrate. A comparison with Table 2 shows that our investigations fit the results obtained by other authors (33-35) for the hydrated form.

Glucose results for the non-hydrated form are shown in Figure 7. The results for a non-hydrated form are shown in Table 2. Our results are in excellent agreement with the results in (36), and in acceptable agreement with the results in (37) and (38). In the literature, additional lines at 59.3, 96.9, 110.5, 115.9, and 124.5 cm^{-1} are reported (37).

Lactose and glucose show their "fingerprints" without any doubts, but Pluronic F127 shows a different picture. The spectrum is very smooth, and it is difficult to describe it as distinctive of Pluronic. Many polymers exhibit a similar character of the spectrum (19). Only a very weak absorption line at 49.9 cm^{-1} was recognized in our experiment.

Mixtures - additive process

Our results show that spectra of pharmacological mixtures are formed additively. It means that the

spectra of the mixtures can be easily predicted by numeric summation of the results obtained separately for the constituent substances. Figure 9 shows the results for selected compounds (glucose and lactose). Figure 9a and b show spectra for glucose and lactose measured separately. Figure 9c presents the result of measurements for the tablet with 10% percent of glucose and lactose (together) with the weight ratio of 50/50. The rest of the tablet, 90%, consists of PTE powder. As can be seen, a few specific absorption lines for considered compounds can be easily recognized in glucose and lactose and they can be recognized in the mixture. Morever, the numerical summation process gives almost the same result. The positions of the absorption lines occupy exactly the same places on the wavenumber axis. Only the value of absorbance is different. This is a consequence of differing compositions of

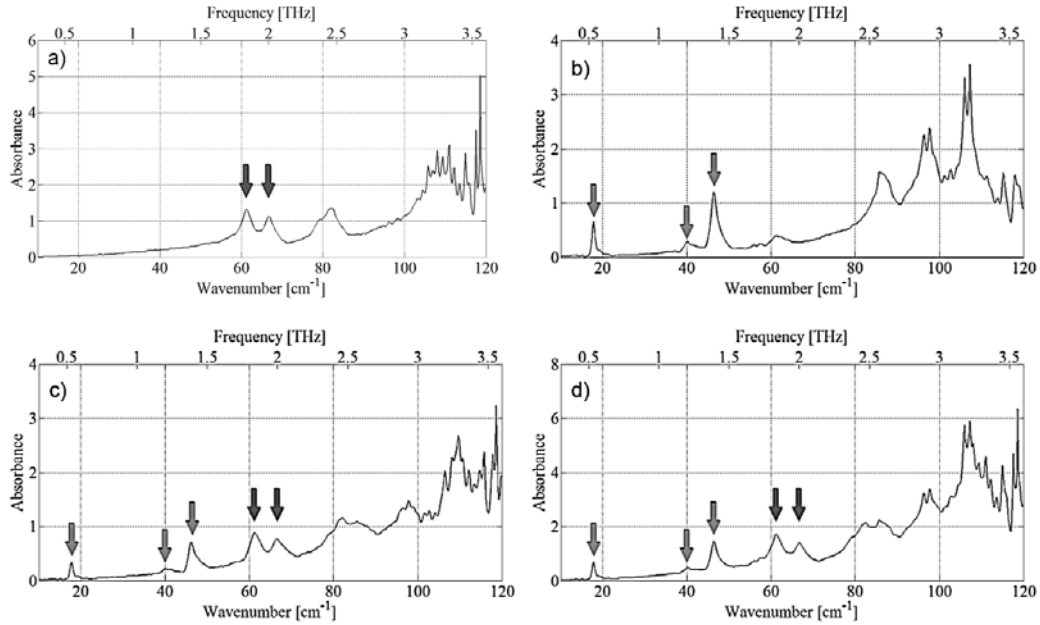

Figure 9. a) glucose (hydrated), b) lactose, c) glucose and lactose mixed in one tablet with PTE powder (90%) in the weight ratio 50/50, d) numerical sum of two signals – glucose and lactose (a + b)

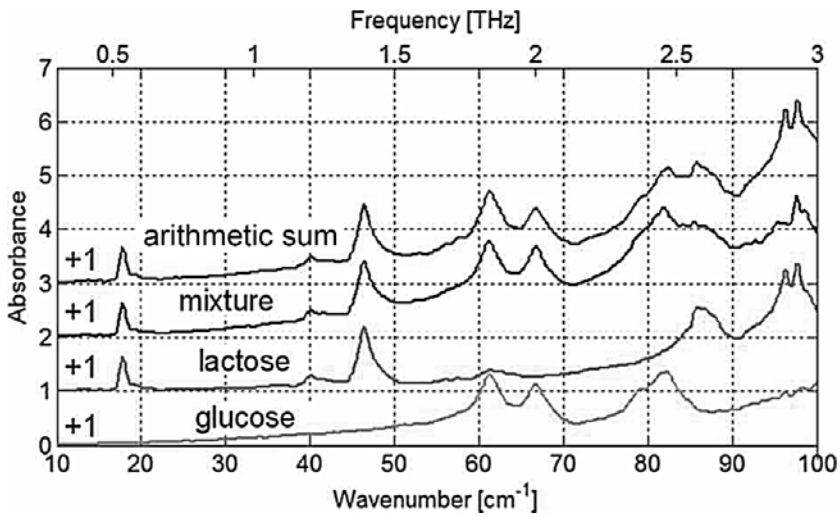

Figure 10. Summarized results in one figure. From bottom to the top: glucose (hydrated), lactose, glucose and lactose mixed in one tablet with PE powder (90%) in the weight ratio 50/50, numerical sum of two signals – glucose and lactose. Spectra are vertically offset for clarity

glucose/lactose in tablets a), b) and c). The result presented in Figure 9d is promising. As can be seen, the summation process does not involve any distortion in the positions of the absorption lines. The summarized results are shown in Figure 10.

Recognition of compounds in mixtures

The idea of combining two or more drugs with complementary modes of action is to produce additivity of the desired therapeutic effect, but not of the side effects. A mixture of fenofibrate (or statin) with aspirin plays an important role in the development of an improved drug delivery system for primary and secondary prevention of coronary heart disease. It also influences the prevention of complications in diabetic patients. In our research, we prepared suitable physical mixtures with aspirin and ketoconazole used in pharmaceutics. Figures 11-13 present the terahertz spectra for physical mixtures of fenofibrate with aspirin and two mixtures of lovastatin with aspirin obtained in different weight ratios.

Figure 11 shows the spectrum of the aspirin with fenofibrate mixture in the ratio 44 : 56, respectively. As can be seen, the aspirin and fenofibrate spectral lines mutually merge. Comparing absorbance of fenofibrate (Fig. 4c) and aspirin (Fig.

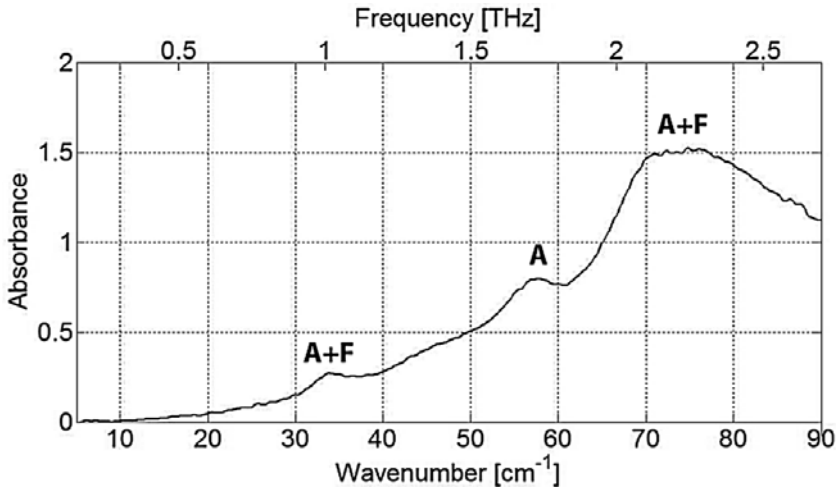

Figure 11. Terahertz spectrum for physical mixture of fenofibrate and aspirin in the weight ratio 0.44/0.56. A – contribution of aspirin, F – contribution of fenofibrate

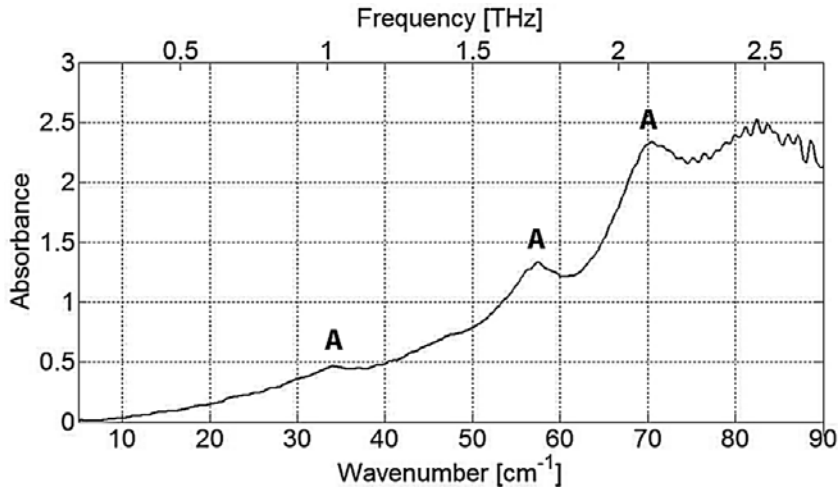

Figure 12. Terahertz spectrum for physical mixture of lovastatin and aspirin in the weight ratio 27.8/72.2. A – contribuiton of aspirin, contribution of lovastatin is overlapped due to higher concentration of the aspirin (cf. Fig. 13)

5b) we have concluded that aspirin has a decisive influence on the absorption peak at 33.9 cm^{-1} (with some influence of fenofibrate). A similar situation can be noted for the second peak at 57.4 cm^{-1}. The last absorption detail around 72.5 cm^{-1} is broadened due to the convolution of two absorption lines: 69.5 cm^{-1} (aspirin) and 76.0 cm^{-1} (fenofibrate).

Another example is shown in Figure 12. The aspirin spectral lines (A) definitely overlap the lovastatin spectrum. In this case the aspirin spectrum is much stronger. The spectrum of aspirin reaches the 3.0 value of absorbance comparing to lovastatin - only 1.7 (see Figs. 5b and 4d), but in this case, the percentage of aspirin in the mixture is much higher. The last absorption feature around 82.6 cm^{-1} is broadened due to the convolution of two absorption lines: 81.9 cm^{-1} (aspirin) and 85.5 cm^{-1} (lovastatin).

A better situation for lovastatin is observed in Figure 13. But in this case there is as much as 83.6% of lovastatin in the mixture with aspirin. The last absorption feature around 84.2 cm^{-1} is broadened due to the convolution of two absorption lines: 81.9 cm^{-1} (aspirin) and 85.5 cm^{-1} (lovastatin).

In our studies, a mixture of ketoconazole with Pluronic F127 was analyzed as well - see Figure 14.

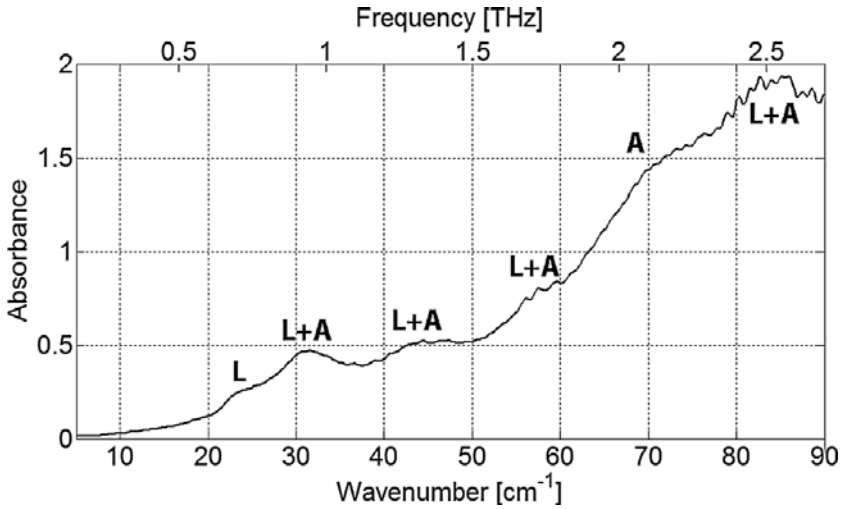

Figure 13. Terahertz spectrum for physical mixture of lovastatin and aspirin in the weight ratio 83.6/16.4. A - contribuiton of aspirin, L - contribuiton of lovastatin

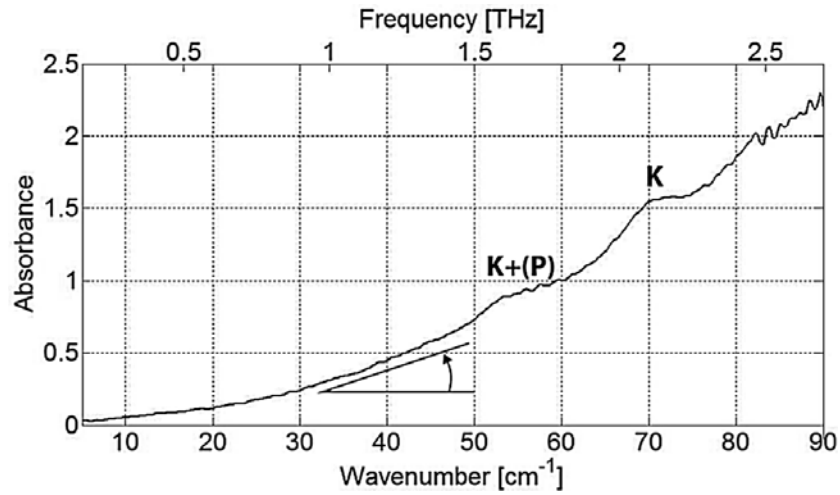

Figure 14. Terahertz spectrum for mixture of Pluronic F127 and ketoconazole in the weight ratio of 50/50. K – contribution of ketoconazole, (P) – weak contribution of Pluronic. The angle of the slope is indicated (explanation in the text)

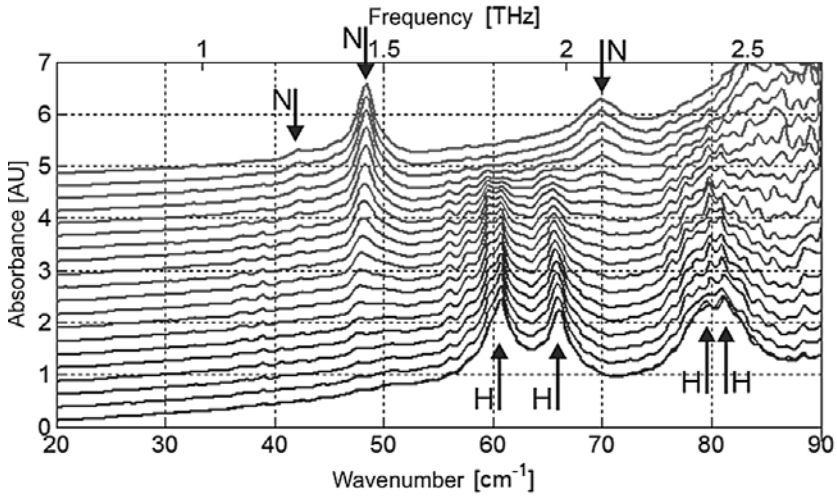

Figure 15. Hydration effect in glucose. Absorption peaks are indicated for both hydrated and non-hydrated forms: H – hydration effect, N – non-hydrated molecule absorption lines. Spectra are vertically offset at 0.25 (AU) for clarity

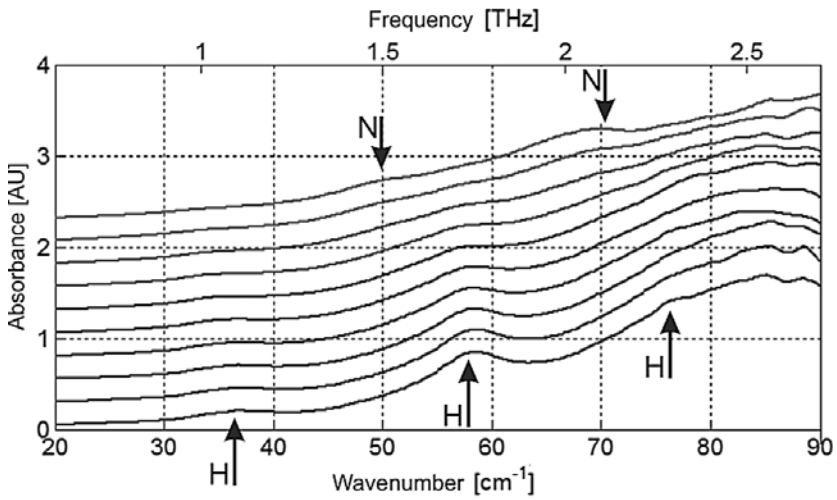

Figure 16. Effect of the drying on hydrated acyclovir (low characteristics). The spectrum of the dry compound is visible at the top of the figure. Spectra are vertically

There is observed an advantage of ketoconazole absorption comparing to Pluronic F127 in the mixture of 50/50. Comparison of both separate spectra (see Figure 5c and Figure 8) reveals that ketoconazole is characterized by much more recognized spectral details than Pluronic. Perhaps in the case of the spectral detail at 53.2 cm^{-1} it can be observed some weak influence of Pluronic on the mixture spectrum indicated in Figure 14 with K + (P). Despite the relatively lower amount of Pluronic in the sample (50% compared to the result presented in Fig. 8) the slope of the characteristic is higher than that in Figure 5c (pure ketoconazole). The influence of Pluronic is obvious.

The spectral details of the mixture spectra are collected in Table 3. All values of the absorption peaks were determined using the second derivative method.

Polymorphism

Many bio-components when exposed to water may form hydrates and, on the contrary, hydrates may lose their water under high temperature or low humidity. The possibility of hydrate formation and dehydration can occur during manufacturing, for example, during several unit operations, such as drying, milling, tableting or during storage. It is important to understand the dehydration behavior of an

Table 3. Collected results of the mixture spectral measurements in terahertz region.

Compound	Absorption line (cm^{-1})
Mixtures	
Fenofibrate/Aspirin 44/56	33.9, 57.4, 72.5
Lovastatin/Aspirin 27.8/72.2	33.9, 57.4, 70.0, 82.6
Lovastatin/Aspirin 83.6/16.4	23.5, 31.0, 44.4, 57.6, 70.4, 84.2
Pluronic/Ketoconazole 50/50	53.2, 70.2

API to get an insight into the dehydration mechanisms encountered during processing, which makes it possible to monitor and control the production process (17, 32, 37, 39).

To show how the effect of polymorphism can influence the shape of the spectrum characteristic in the terahertz region, we chose two compounds: glucose and acyclovir. The results of the hydration process are shown for both compounds. This is also referred as a pseudo-polymorphism. Figure 15 shows the effect on the glucose. The hydrated form is heated from room temperature to 120°C. As can be seen, some absorption lines (indicated as "H") disappear at the higher temperature, when the compounds are dried. Instead, two lines (indicated as "N") appear but in different parts of the observed spectral band.

Figure 15 shows, how clear and easy it is to recognize the pictures of hydrated and non-hydrate forms of chemical compounds - here hydrated and non-hydrated glucoses. The absorption lines appear in completely different places of the frequency axis: in the case of hydrated glucose at 61.3, 66.5, and 81.9 cm^{-1}; in the case of non-hydrated one at 42.7, 48.8, 70.0, and 84.7 cm^{-1} (see Table 2). The presence or absence of water molecules in the molecular structure of the crystaline glucose influence strongly the spectral characteristics. Both components, hydrated and non-hydrated, leave clear traces ("fingerprints") in the spectral characteristics.

Another example of the hydration effect is shown on acyclovir. Figure 16 shows the process of drying. Family of the characteristics at the bottom of the figure shows the beginning of the process from room temperature to approximately 120°C.

Absorptions lines 36.5, 58.5, and 77.5 cm^{-1} create the "fingerprints" for hydrated acyclovir, and two lines 49.0, 70.4, cm^{-1} create "fingerprints" for the non-hydrated form. As in the case of a hydration effect in glucose, the spectral picture for acyclovir is easy to recognize too.

CONCLUSIONS

As has been shown, the pharmaceutical compounds and molecules under investigation have specific "fingerprints" in the terahertz spectroscopy band. The compounds can be easily recognized in the THz region. We used a terahertz time-domain spectroscopy technique to achieve our goal. To improve the measuring methodology, we indicated the absorption lines using the second derivative method applied to the signals obtained. This method can be used to enhance absorption lines e.g., for automatic absorption lines recognition.

We investigated different active pharmaceutical ingredients: (famotidine, ranitidine, fenofibrate, lovastatin, simvastatin, aspirin, ketoconazole) and excipients (lactose, glucose, Pluronic). All measured compounds showed clear spectral details. In this way, we have shown that the spectral images can be easily assigned to individual components. The details of most of the components are so easy to recognize that they are visible even in mixtures of the compounds.

We showed the spectra of typical mixtures used as drugs. We investigated mixtures of fenofibrate/aspirin, and lovastatin/aspirin - (excipient + API) in typical proportions such as 27.8/72.2 and 83.6/16.4, respectively. A Pluronic plus ketoconazole mixture in weight proportion of 50/50 was investigated as well. All individual absorption lines were preserved in the spectra of mixtures. Moreover, when the spectra of separately measured compounds were numerically added, then the same spectra were obtained as for real mixtures. As it was shown, the spectra exhibit additive properties.

The terahertz technique is a useful tool for detection of the parasitic process of drug hydration, which is a typical problem in the pharmaceutical industry occurring during the manufacturing process. We examined two substances - glucose (a

typical excipient in many forms of drugs), and acyclovir (API). In our investigations we have shown the process of water release with the temperature increase.

We have shown in this paper that the terahertz technique can be applied for the monitoring and controlling of the technological process not only by spectral investigations of the drugs, but also by terahertz technique of imaging. A tablet of ketanol was selected as a test sample for the THz imaging. The pictures of cross-sections of the tablet for different penetration depths were obtained. As was shown, the defects of the tablet were easily recognized.

Development of the terahertz technique and its diverse range of applications in recent years, makes it very probable that both terahertz spectroscopy and terahertz imaging will become commonly established techniques in pharmaceutical research, providing excellent opportunities for the pharmaceutical sciences and industry.

Acknowledgment

The authors thank Professor Renata Jachowicz for inspiration.

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SIMPLE, SENSITIVE AND SELECTIVE SPECTROPHOTOMETRIC ASSAY OF NAPROXEN IN PURE, PHARMACEUTICAL PREPARATION AND HUMAN SERUM SAMPLES

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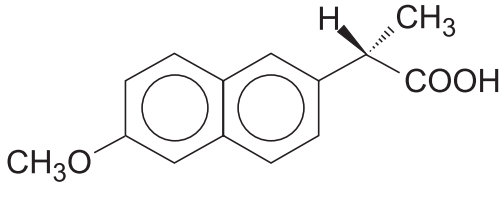
Abstract: Two simple, rapid and sensitive spectrophotometric methods have been developed for the determination of naproxen in pure, pharmaceutical preparation and human serum samples. These methods are based on the formation of yellow ion-pair complexes between naproxen and two sulfophthalein acid dyes, namely bromocresol green (BCG method) and bromothymol blue (BTB method). The resulting complexes were measured at 424 nm (BCG method) and at 422 nm (BTB method). The effects of variables such as reagent concentration and reaction time were investigated to optimize the procedure. Beer's law was obeyed in the concentration range of 10-105 µg/mL and 5-85 µg/mL and the detection limits were found to be 0.347 and 0.31 µg/mL for BCG and BTB methods, respectively. The developed methods have been successfully applied for the determination of naproxen in bulk drugs, pharmaceutical formulations and human serum samples with good accuracy and precision. The results are comparable to those of reference methods, and hence are recommended for quality control and routine analysis.

Keywords: naproxen, spectrophotometry, bromocresol green, bromothymol blue

Naproxen (NAP) [(+)-(*S*)-2-(6-methoxynaphthalen-2-yl) propanoic acid], is a non-steroidal anti-inflammatory drug (NSAID). Due to an aryl acetic structure, naproxen exhibits analgesic and antipyretic properties (Scheme 1). Only the (+) enantiomer possesses anti-inflammatory properties. Naproxen is used for the reduction of moderate to severe pain, fever, inflammation, rheumatoid arthritis, musculoskeletal disorders and gout.

Anti-inflammatory effects of naproxen are generally thought to be related to its inhibition of cyclooxygenase and consequent decrease in prostaglandin concentrations in various fluids and tissues (1, 2). Naproxen is generally well tolerated and the most common side effects that have been reported are: gastrointestinal complaints, headache, vomiting, diarrhea, constipation, decreased appetite, rash, dizziness and drowsiness. In some cases, gastric and also bleeding ulcers could be produced and in a few cases renal failure, hepatic injury, urticaria, ecchymosis and vasculitis have been reported. Naproxen is rapidly and completely absorbed after oral administration and it is the predominant species in serum, with a therapeutic range of 30 to 90 µg/mL (3). NSAIDs may cause an increased risk of serious cardiovascular thrombotic events, myocardial infarction, and stroke, which can be fatal. This risk may increase with duration of use. Due to its extortionary use as non-prescription drug, naproxen has been detected in surface water, groundwater, wastewater and even in drinking water in the range from ng/L to several g/L (4), therefore, it is required to develop a simple, effective, rapid and accurate method that can be used in routine quality control. Naproxen has been determined by several analytical methods like: HPLC (5-8), HPTLC (9), TLC/UV (10), LC-MS/MS (11, 12), capillary electrophoresis (13, 14), fluorimetric methods (15, 16), chemiluminescence (17-19), voltametry (20, 21), liquid phos-



Scheme 1. Chemical structure of naproxen

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phorimetry (22), solid-phase microextraction coupled to liquid chromatography (23), synchronous spectrometry (24), and spectrophotometry (25-30). Several chromatographic methods have been reported for single and simultaneous determinations of naproxen in tablets (31), human blood plasma (32) and urine (33). Although HPLC methods are highly sensitive and specific, they are considered expensive.

Spectrophotometry is considered as the most convenient analytical technique in pharmaceutical analysis because of its inherent simplicity and availability in most quality control and clinical laboratories (34-37). In this work, we developed two simple, rapid, accurate, precise, sensitive and less time consuming spectrophotometric methods for quantitative determination of naproxen in pure, pharmaceutical preparation and human serum samples. These methods are based on the formation of ion pair complex between naproxen and sulfone phthalein acid dyes, namely bromocresol green (BCG) and bromothymol blue (BTB).

MATERIALS AND METHODS

Stock standard solutions of naproxen

A stock standard solution (200 µg/mL) was prepared by dissolving accurately weighed 20 mg of pure naproxen in methanol and diluting to the mark with the same solvent in a 100 mL calibrated flask. This stock solution was diluted appropriately with methanol to obtain suitable working solutions. Freshly prepared solutions were always employed.

Stock standard solutions of reagents

Stock solution of BCG and BTB (100 µg/mL), were prepared by dissolving 10 mg of reagents in methanol and diluting to the mark with double distillated water in a 100 mL calibrated flask. The acid dye reagents were stable for one week.

Apparatus

All absorbance measurements and spectral runs were made on a RAYLEIGH UV-1800 single beam spectrophotometer (BRAIC, Beijing, China) with 1

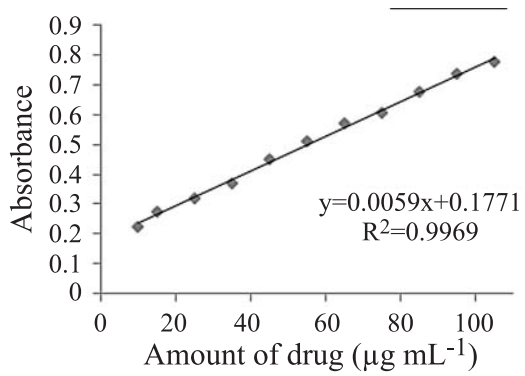

Figure 1. Calibration curve of naproxen with BCG

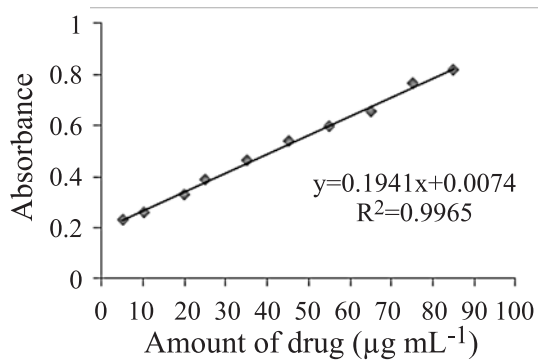

Figure 2. Calibration curve of naproxen with BTB

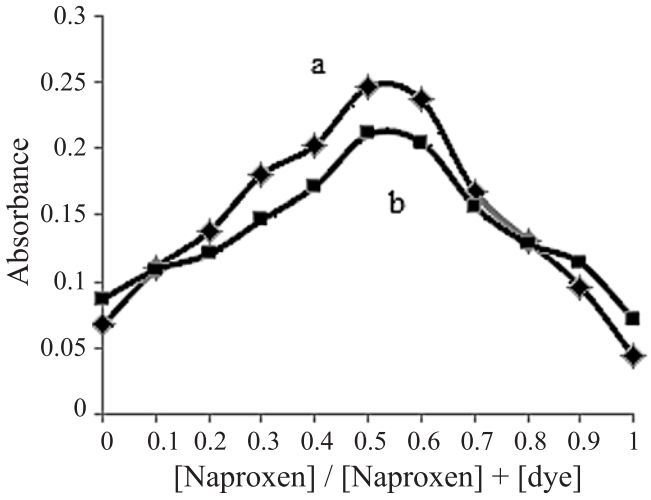

Figure 3. Job's plot of continuous variations: reaction stoichiometry between naproxen and (a) BTB, (b) BCG

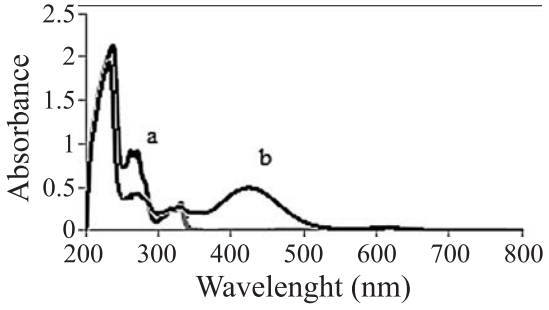

Figure 4. Absorption spectrum of (a) pure naproxen and (b) ion-pair complex of naproxen with BCG against reagent blank

cm matched quartz cells. The pH measurements were carried out with a Metrohm 827 pH lab pH meter.

Reagents

All the chemicals and reagents used were of analytical grade (Merck) and used without further purification. Naproxen was supplied as a gift sample from Sobhan Pharmaceutical (Rasht, Iran). Tablet formulation of naproxen was procured from a local pharmacy for analysis.

General procedures

Procedure for calibration curves

BCG method: Aliquots equivalent to 5-105 µg/mL naproxen were trasfered into a series of 5 mL volumetric flasks. To each flask, 1 mL of 50 µg/mL of BCG solution and 1 mL of phosphate buffer solution (pH = 3.5) were added and made up to mark with methanol and distillated water, then, were left to stand for 10 min at room temperature (20 ± 5°C). The absorbance the of the yellow colored complex were measured at 424 nm against a blank reagent prepared in the same way without addition of the naproxen. To obtain the standard calibration graph, plot of the values of absorbance against the drug concentration (Fig. 1) was used.

BTB method: Aliquots equivalent to 5-85 µg/mL naproxen were transfered into a series of 5 mL volumetric flasks. To each flask, 1 mL of 50 µg/mL of BTB solution and 1 mL of phosphate buffer solution (pH = 3.0) were added and made up to mark with methanol and distillated water, then, left to stand for 10 min at room temperature (20 ± 5°C). The absorbance of the yellow colored complexes were measured at 422 nm against a blank reagent prepared in the same way without addition of naproxen. To obtain the standard calibration graph, plot of the

values of absorbance against the drug concentration (Fig. 2) was used.

Procedure for the assay of tablets

For the analysis of naproxen in tablets by the proposed methods, ten tablets of naproxen were weighed and pulverized into a fine powder. An accurately weighed portion of the powdered tablets equivalent to 250 mg of naproxen was transferred into 100 mL beaker and was dissolved in the least amount of methanol, filtered through a Whatmann No. 41 filter paper, washed with methanol into a 100 mL calibrated flask and diluted to volume with methanol. Solutions of working range concentration were prepared by proper dilution of this stock solution with methanol and followed the above procedure for the analysis. The drug content of the tablets formulation was then calculated (Table 1). For further confirmation, the standard addition technique was applied to test the reliability and recovery of the proposed methods, in which variable amounts of the drug were added to the previously analyzed portion of pharmaceutical (Table 2).

Procedure for spiked serum

The proposed methods have been successfully applied for the determination of naproxen in human blood serum samples. The results were obtained from four replicate measurments of serum samples containing naproxen and indicated that the proposed methods were effective for the determination of naproxen in human blood serum samples (Table 3).

Stoichiometric relationship

The composition of ion-pair complexes were established by applying Job's method of continuous variations; a 1×10^{-4} M standard solution of naproxen and 1×10^{-4} M solution of reagents (BTB, BCG) were used. A series of solutions were prepared in which the total volume of drug and reagent was kept at 1.0 mL. The reagents were mixed in various proportions and diluted to volume in a 5 mL calibrated flask with the appropriate solvent following the

Table 1. Application of the proposed methods for the determination of naproxen in tablet dosage form.

Method	Naproxen labeled amount (mg)	Found (mg)	Recovery (%)	RSD[a] (%)
BCG	250	251.41	100.56	0.81
BTB	250	249.18	99.67	0.58[a]

Mean of four determinations.

Table 2. Determination of naproxen in tablet dosage form using the standard addition technique.

Sample	Taken (µg/mL)	Added (µg/mL)	BTB		BCG	
			Found[a] (µg/mL)	Recovery (%)	Found[a] (µg/mL)	Recovery (%)
Naproxen (250 mg)	10	0	10.14	101.40	9.84	98.40
		2	12.38	102.17	11.91	99.25
		4	13.76	98.28	13.69	97.78
		6	16.26	101.62	16.19	101.19

[a] Mean of four determinations.

Table 3. Application of the proposed methods for the determination of naproxen in human blood serum.

Method	Added (µg/mL)	Recovery a (%)	RSD (%)	Difference (%)
BCG	5	101.14	0.22	1.14
	10	102.00	0.76	2.00
BTB	5	101.45	0.70	1.45
	10	98.82	0.67	-1.18

[a] Mean of four determinations.

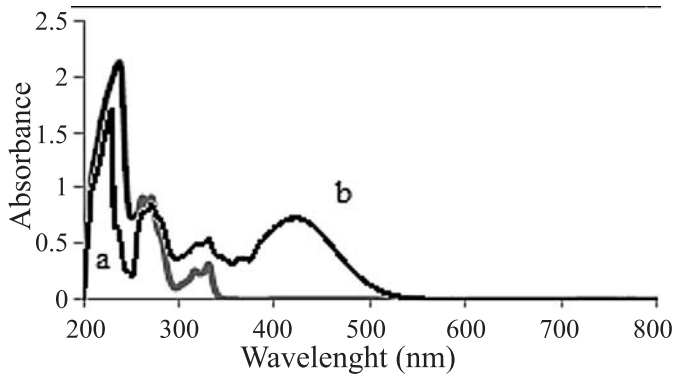

Figure 5. Absorption spectrum of (a) pure naproxen and (b) ion-pair complex of naproxen with BTB against reagent blank

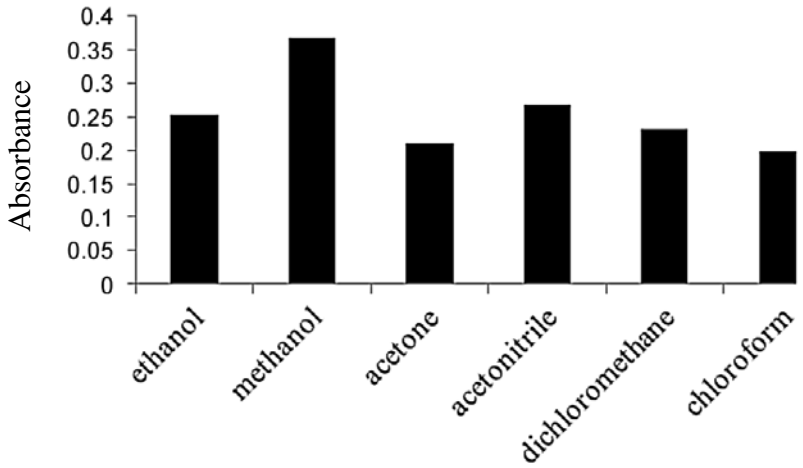

Figure 6. Effect of different solvents on the formation of colored product (BCG method)

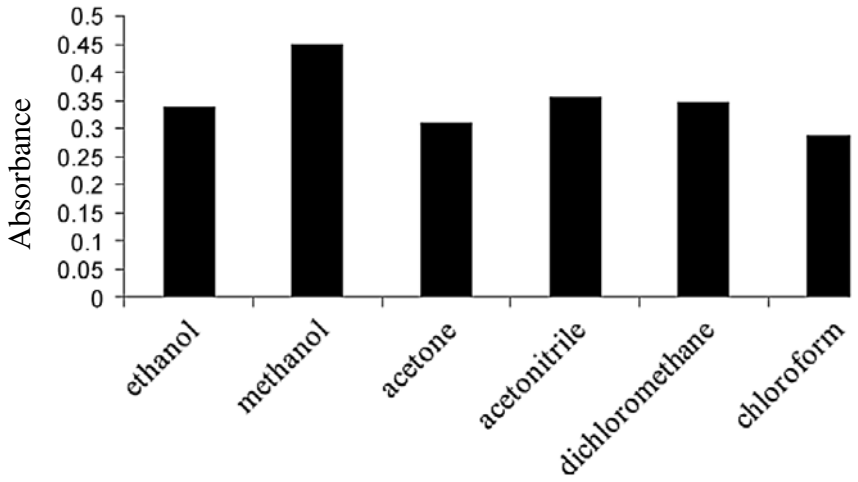

Figure 7. Effect of different solvents on the formation of colored product (BTB method).

above mentioned procedure. The plot reached a maximum value at a mole fraction of 0.5, which indicated that a 1 : 1 (drug : dye) ion-pairs are formed through the electrostatic attraction between positive protonated drug and BTB ot BCG anions.

RESULTS AND DISCUSSION

Absorption spectra

The absorption spectra of the ion-pair complexes, formed between naproxen and each of BPB and BCP were measured at 200-800 nm against the blank solution prepared under the same conditions (Figs. 4 and 5). The complexes showed maximum absorbance at 424 and 422 nm for naproxen-BCG and naproxen-BTB complex, respectively. The measurements were made at these wavelengths for tablets and human serum samples. The absorption band of the reagent showed λ_{max} at 612 and 592 nm for BCG and BTB, respectively.

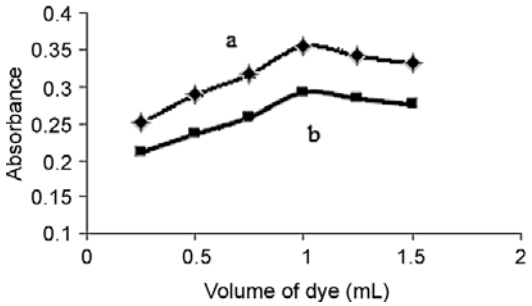

Figure 8. Effect of dye concentration on the formation of colored product with (a) BTB and BCG (b)

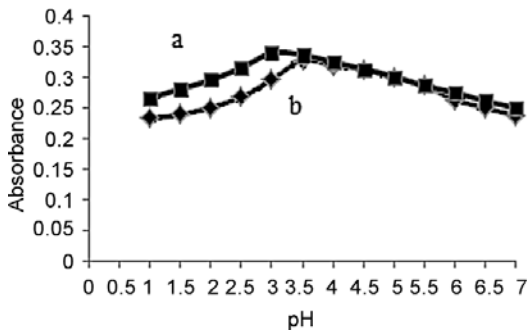

Figure 9. Effect of pH on the formation of colored product with (a) BTB and BCG (b)

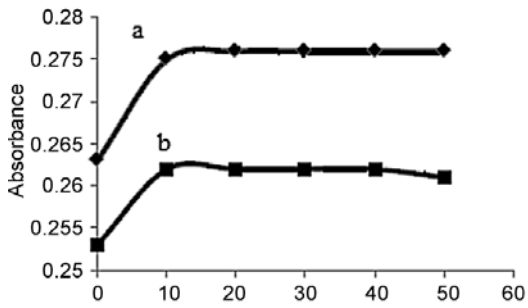

Figure 10. Effect of time on the formation of colored product with (a) BTB and BCG (b)

Optimum conditions for complex formation

In order to establish the optimum conditions necessary for a rapid and quantitative formation of the colored product with maximum stability and sensitivity, the absorbance of a series of solutions was measured by varying one parameter while keeping the others constant.

Selection of solvent

The effect of several solvents such as ethanol, methanol, acetone, acetonitrile, dichloromethane and chloroform were investigated on the absorbance of the yellow color complexes. It is found that the reaction mixture is becoming turbid when diluted with distilled water. The difference in absorbance values with other solvents are shown in Figures 6 and 7. It is apparent from the figures that the highest absorbance was obtained in methanol medium. Therefore, methanol was selected as the best solvent.

Effect of reagent concentration

The effect of the dye concentration on the intensity of the color developed at selected wavelengths was tested using different volumes of the reagents. The results shown that 1 mL of BCG and BTB were found to be optimum for these proposed methods and excess of these dyes do not affect the color of the complexes or the absorbances (Fig. 8).

Effect of pH

The influence of pH of buffer solution on the development and stability of the color using phosphate-HCl buffer solution was studied over the pH range 2.0-7.0. The maximum color intensity was observed in the pH of 3.0 and 3.5 for naproxen-BTB and naproxen-BCG complex, respectively (Fig. 9). Moreover, the optimum volume of buffer solution added to 5 mL to give constant absorbance value was also studied and found to be 1 mL.

Effect of reaction time

The effect of time on the formation and stability of the ion-pair complexes was studied by measuring the absorbances of these complexes at increasing time intervals. At the beginning, the absorbance

Table 4. Analytical parameters for the determination of naproxen by the proposed methods.

Parameter	Naproxen - BCG	Naproxen - BTB
λ_{max} (nm)	424	422
Beer's law limits (µg/mL)	10-105	5-85
ε (L/mol cm) pH	2.59×10^3 3.5	2.969×10^3 3.0
Regression equation (y)[a]	y = 0.005x + 0.177	y = 0.007x + 0.194
Correlation coefficient (r^2)	0.9969	0.9965
RSD %	0.76	0.54
LOD (µg/mL)	0.347	0.312
LOQ (µg/mL)	1.158	1.02

[a] $y = a + bx$, where x is the concentration in µg/mL.

Table 5. Evaluation of the accuracy and precision of the proposed methods for naproxen determination.

Method	Added (µg/mL)	Recovery a (%)	RSD (%)	Er (%)
BCG	10	102	0.99	2.00
	35	99.94	99.94	-0.06
BTB	5	98.4	1.07	-1.6
	20	101.1	0.67	1.10

[a] Mean of four determinations.

increased gradually along with the time. After 10 min, it achieved stability and remained basically unaltered (Fig. 10).

Effect of sequence of additions

The order of reagent addition was very important; changing the order produced low result. The most favorable sequence is 'drug-reagent-buffer-solvent' for the complete color development and the highest absorbance at the recommended wavelength. Other sequences needed longer time and produced lower absorbance values.

Stability of ion-pair complexes

The stability of the ion-pair complexes formed between drug and acidic dye was evaluated. The formation of the ion-pair complexes was rapid and the yellow color products were stable for 3 days for naproxen-BTB and naproxen-BCG without any change in color intensity in dark and room temperature.

Effect of interferences

The effects of common excipients and additives were studied for their possible interferences in the assay of naproxen. The results revealed the fact that no significant interference was observed from the excipients, such as glucose, fructose, sucrose, lactose and starch commonly present in pharmaceutical formulations. This shows that the methods are applicable in the case of pharmaceutical preparations of the naproxen.

Method validation

The proposed methods have been extensively validated in terms of linearity, accuracy, precision, limits of detection (LOD) and quantification (LOQ) (Table 4). The accuracy was expressed in terms of percent recovery of the known amount of the standard drugs added to the known amount of the pharmaceutical dosage forms. In order to determine the accuracy and precision of the methods, solutions containing two different concentrations of the studied drug were prepared and four replicates determinations, covering the usable concentration range, were carried out for pure form and the pharmaceutical preparation of naproxen. The analytical results obtained for this investigation are summarized in Table 5. The low values of RSD % indicate good precision and reproducibility of the proposed methods. The average percent recoveries obtained were quantitative, indicating good accuracy of the meth-

Table 6. Comparison of the proposed method with existing spectrophotometric methods for the determination of naproxen in pharmaceutical formulations.

Reagent/s	λ_{max} (nm)	Linear range (μg/mL)	Molar absorptivity (L/mol cm)	RSD (%)	Ref.
3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH)	500	1.00-12.0	1.15×10^4	1.3	(38)
Oxidation with alkaline potassium permanganate	608	0.4-2.8	2.3×10^4	1.32	(39)
Niacinamide as hydrotropic solubilizing additive	331	50-250	-	1.108	(40)
HPLC method	230	5.0-150	-	3.7	(41)
Atomic absorption spectrometry method based on reaction with copper (II) chloride and cobalt (II) chloride	324.8 240.7	2.5-25.0 3.5–23.5	- -	0.13 0.24	(27)
Stability-indicating spectrophotometric method	332	150–645		0.9	(42)
BCG	424	10–105	2.59×10^3	15	In this work
BTB	422	5–85	2.969×10^3	15	In this work

ods. The performance of the proposed method was compared with that of other existing UV-visible spectrophotometric methods (Table 6).

CONCLUSION

Unlike HPLC method, the spectrophotometric procedure is simple and is not high cost. The main purpose of this study was to establish two simple, economic and rapid UV/Visible spectrophotometric methods for determination of naproxen in pure, tablet dosage form and human serum samples. The reagents utilized in this work are cheap and available and the proposed methods do not include any crucial reaction conditions. Also these methods were applied directly to the analysis of pharmaceutical dosage forms and serum samples without the need for separation or extraction steps prior to drug analysis. The high recovery percentage and low relative standard deviation reflect the high accuracy and precision of these proposed methods. Therefore, the proposed methods can be successfully applied for the routine analysis of naproxen.

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THE MEASUREMENT OF ANTIOXIDANT CAPACITY AND POLYPHENOL CONTENT IN SELECTED FOOD SUPPLEMENTS*

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Abstract: Oxidative stress (OS), defined as a disturbance in the balance between the production of reactive oxygen species (ROS) and antioxidant defenses, can result in the development of many serious diseases like diabetes or cancer. Moreover, the role of oxidative stress in the acceleration of the aging process is also confirmed. ROS are constantly produced in the natural biochemical processes, mainly during cellular respiration. Their enhanced production may be the result of e.g., an inappropriate diet high in saturated fats, low in fiber, fruits and vegetables, insufficient physical activity or smoking. To prevent oxidative stress, besides changes in life style, the additional supplementation of antioxidants is proposed. On the Polish market, the number of food supplements with declared antioxidant activity is still increasing. However, their antioxidant properties are rarely confirmed experimentally. The aim of our study was to determine the antioxidant potential of selected dietary supplements available on the market and recommended in chronic fatigue syndrome. The antioxidant potential was measured using four methods: FRAP, ORAC, HORAC, EPR/DPPH. Moreover, the content of polyphenols in the dietary supplements was also determined.

Keywords: FRAP, ORAC, HORAC, EPR/DPPH, pharmaceuticals, antioxidant capacity

Oxidative stress (OS) is a homeostatic imbalance between the natural antioxidant defenses and the production of reactive oxygen species (ROS) (1). ROS are produced in the natural biochemical processes, but in excessive amounts and without sufficient antioxidant defenses what can cause damage to all components of cells: lipids, proteins and DNA (2). Several epidemiological studies have shown that degenerative diseases, including cancer, cardiovascular, neurodegenerative diseases and immune dysfunction, which are associated with increased ROS activity lead to the OS (3-5). Recent studies report that high consumption of antioxidant food products like vegetables, fruits and beverages, for example, tea, wine and cocoa, can reduce the oxidative stress and reduce the risk of chronic diseases (6). These food products are a rich source of antioxidant compounds like vitamins (C and E), selenium and carotenoids, such as β-carotene, lycopene, lutein and polyphenols (7).

Inappropriate lifestyle, exposure to environmental pollutants, toxins, tobacco smoke, artificial chemicals and a diet poor in antioxidants may induce an abnormal increase of ROS production and/or a decrease in antioxidant defenses that could alter an homeostatic balance state and cause OS. Healthy lifestyle associated with low exposure to toxins and a healthful, balanced diet supplies the body with sufficient nutrients, e.g., phytochemicals, and leads to the lower risk for many diseases (8). Supplements with compounds such as vitamins, minerals, essential fatty acids, phytochemicals can enrich the body's internal environment to fortify cellular protection, regeneration and support detoxification processes (9).

There are a number of commercial dietary supplements based on fruits and vegetables with the declared antioxidant activity, which can be defined as a protection against OS (10). As manufacturers declare, dietary supplements contain compounds with proven antioxidant properties, which help in maintaining good health, delay the aging process and reduce the risk of many diseases. However, there are also dietary supplements without declared antioxidant

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properties which are used in the prevention and treatment of many diseases like, for example, digestion.

Dietary supplements are prepared from different parts of plants, have different chemical compositions and different concentrations of active compounds, therefore they vary widely in their antioxidant properties. Furthermore, most manufacturers offer residual information about dietary supplements composition and their labels include no data on their antioxidant activity. Because the dietary supplements are used by man as a source of natural antioxidants, their antioxidant properties should be known, standardized and controlled. The standardization of antioxidant supplements would allow to recommend efficacious doses and to ensure that these products may have a beneficial effect. To date, there are only a limited number of studies on this subject (11, 12).

On the Polish market there are many dietary supplements with declared antioxidant activity and many with potential antioxidant activity. However, their antioxidant properties are rarely confirmed experimentally. In the literature there are many analytical methods for determining antioxidant properties of foods and ingredients of different plants. The objective of this work was to evaluate the antioxidant activity *in vitro* of commercial dietary supplements using different methods: FRAP (Ferric Reducing Ability of Plasma), ORAC (Oxygen Radical Absorbance Capacity), HORAC (Hydroxyl Radical Averting Capacity) and DPPH-EPR (diphenyl-1-picrylhydrazyl radical scavenging with the use of electron paramagnetic spectroscopy) assays. The total content of polyphenols, as the most frequently occurring phytochemicals in the dietary supplements, was also determined.

EXPERIMENTAL

Dietary supplements

Dietary supplements were a kind gift from Gama-Tech. Since they are mainly a mixture of various herbal extracts or other components, their detailed composition as indicated by the manufacturer is presented in Table 1.

Sample preparation

To 500 mg of each supplement 10 mL of ethanol-water (1 : 1, v/v) mixture was added. After 40 min of shaking, the samples were centrifuged and the supernatants were taken for analysis.

Antioxidant activity determination

ORAC assay

The ORAC-fluorescein (ORAC-FL) assay was based on the procedure of Ou et al. (13). All solutions used were prepared daily in PBS (phosphate-buffered saline), pH 7.4. The samples solutions were further diluted with PBS. For measurements, 70 µL of PBS, 30 µL of PBS diluted sample or, in case of a blank, 30 µL of PBS buffer and 100 µL of 112 nM sodium fluorescein solution were mixed in a well and thermostated for 15 min at 37°C. Then, 100 µL of 48 mM AAPH solution was added and fluorescence was measured every 60 s for 90 min with F-7000 Fluorescence Spectrophotometer (Hitachi) equipped with a Micro Plate Reader accessory. The excitation wavelength was 485 nm, the emission wavelength was 520 nm. ORAC values in Trolox equivalents (TE [µmol/L g]) were calculated using the standard curves, prepared in parallel with measurements, with Trolox concentration in the range 6.25-100 µM. All experiments were performed in triplicate.

HORAC assay

The HORAC assay was performed according to Ou et al. (14), using F-7000 Fluorescence Spectrophotometer (Hitachi) equipped with a Micro Plate Reader accessory with excitation wavelength set at 485 nm and the emission wavelength set as 520 nm. To each well 70 µL of PBS and 30 µL of a PBS-diluted sample (2-10-fold) or standard solution was pipetted, then 100 µL of 112 nM fluorescein solution was added and all reagents were mixed thoroughly. In the next step, 50 µL of 0.165 µM hydrogen peroxide solution was added, the reagents were mixed well and then 50 µL of the cobalt solution (17.9 mg of cobalt chloride and 23 mg of picolinic acid per 10 mL) was added, and fluorescence was measured every minute for 35 min. Phosphate buffer was used as a blank, and gallic acid concentrations of 800, 600, 400, 200, and 100 µM were used as standards.

FRAP assay

The FRAP assay was performed based on the procedure of Benzi et al. (15). FRAP reagent was prepared by mixing 10 mM 2,4,6-tri[2-pyridyl-s-triazine] (TPTZ), 20 mM ferric chloride and 300 mM (pH = 3.6) acetate buffer in 1 : 1 : 10, v/v/v ratio. Ten µL of sample or standard solution was added to 200 µL of FRAP reagents, the mixture was kept at 37°C and the absorbance reading at 593 nm (Synergy™ Mx microplate reader, Biotec) was taken after 4 min. Where appropriate, the samples were diluted using Millipore water. Standard solutions of ferrous sulfate in the 100-1000 µM range were used for the standard curve preparation (C_{Fe} = -65 + 693 A, where C_{Fe} – ferrous sulfate concentra-

Table 1. Composition of the commercial antioxidant supplements and the other studied samples.

Dietary supplements/ products	Composition of the supplement/product	Recommended daily dose	Declared properties
Acerola extract, powder	Powdered acerola (*Malpighia punicifolia*) extract	No manufacturer recommendation, in practice average dose: 70 mg/day	Antioxidant properties
ALFA AKTIV	Blackcurrant and aronia extract with honey: Vitamin C (996 mg/2 vials), Vitamin B_3 (4.13/2 vials), Selenium (9.04 µg/2 vials), Iodine (23.76 mg/2 vials), polyphenols (8.24 mg/2 vials)	4 vials/day (80 g)	Antioxidant properites
Antiox	Pressed grape extract (150 mg/capsule), *Ginkgo biloba* extract (26.5 mg/capsule), yeast rich in selenium (50 µg/capsule), ZnO (15 mg/capsule), Vitamin C (65 mg/capsule), Vitamin E (10 mg/capsule), β-carotene (5 mg/capsule)	2 capsules/day (780 mg/day)	Antioxidant properties Stimulates immunity and resistivity of the organism.
Chinese Yam	Powdered Chinese Yam (*Dioscorea batatas*)	No specification, in Traditional Chinese Medicine (TCM) minimal recommended dose is 10 g/day	Stimulates immunity and resistivity of the organism.
Detox+	*Uncaria tomentosa (*cv. *Vilcacora)* extract (380 mg/capsule)	1 capsule/day (387.5 mg)	Antioxidant properties
Duolife (Day)	Extract of: acai berry, aloe vera, elderberry, wild rose, grenade, hawthorn, medicago, raspberry noni - indian mulberry ginseng, cranberry	1 vial/day (25 g)	Antioxidant properties. Stimulates immunity and resistivity of the organism.
Duolife (Night)	Extract of: beetroot, goji berries, mulberry - morus alba, milk thistle, nettle	1 vial/day (25 g)	Antioxidant properties. Stimulates immunity and resistivity of the organism.
Floradix	Water solutions (54%) of: carrots, nettle, spinach, couch grass root, fennel, ocean algae, African mallow flower; fruit juice concentrates are 29.4% (pear, grape, black currant, cherry, apples, oranges, beets, lemon yeast, honey, wild rose), Vitamin C (13 mg/ vial), Vitamin B6 (0.4 (mg/vial), Vitamin B12 (0.6 mg/vial), Iron (7.5 mg/vial)	2 vials/day 20 g)	Supplements the daily diet with iron and vitamins. Recommended in order to improve the health and well-being.
Floradix Ochrona Jelit	Water extract of turmeric, peppermint leaves, artichoke leaf, rosemary leaf (8.4 mL/ vial), apple-plum extract (4.5 g/vial), dry extract of turmeric (76 mg/vial), magnesium (125 mg/vial), Vitamin C (13 mg/vial), Thiamine (0.8 mg/vial), Riboflavin (0.9 mg/vial), Vitamin B6 (0.4 mg/vial), Vitamin B12 (0.6 mg/vial), Iron (7.5 mg/vial)	1 vial/day (20 g)	Helps to maintain healthy intestines and proper digestion.
Gano Excel Cordyceps	Powdered mycelium of *Cordyceps sinensis*	2 capsules/day (900 mg)	Improves and strengthens the immunization system. Improves the health of the respiratory system and pulmonary function.

Table 1. Cont.

Dietary supplements/ products	Composition of the supplement/product	Recommended daily dose	Declared properties
Gano Excel Ganoderma	Powdered spores of the Reishi mushroom (*Ganoderma lucidum*m) (275 mg/1 capsule)	2-4 capsules/day (550-1100 mg)	Stimulates immunity and resistivy of the organism
Gano Exel Excellium	Powdered mycelium of the Reishi mushroom (*Ganoderma lucidum*m) (425 mg/1 capsule)	2-4 capsules/day (850-1700 mg)	Helps to support general well being and nurtures the body's natural defenses
ImmunoBooster	Pomegranate extract with ellagic acid (40 mg/2 capsules), acerola extract with natural Vitamin C (80 mg/2 capsules) red raspberry, graviola and arnica extract, blueberry leaf extract	2 capsules/day (120 mg)	Antioxidant properties
Long Energy	Dry extract of *Rhodiola rosea L.* (200 mg/2 tablets), *Cola nitida (Vent.) Schott. et Endl.* (200 mg/2 tablets), powdered *Panax ginseng C.A. Meyer* (120 mg/2 tablets), dry extract of *Ginkgo biloba L.* (90 mg/2 tablets), yeast rich in selenium (105 µg/2 tablets), coenzyme Q10 (30 mg/2 tablets)	2 tablets/day (641 mg)	Antioxidant properties
Mistify	Acai berry extract (*Euterpe oleracea*) (1.60 g/vial), red grape extract (*Vitis vinifera)* (1.86 g/vial), Concorde grape extract (*Vitis labrusca*) (1.86 g/vial), Highbush blueberry extract (*Vaccinum corymbosum*) (1.25 g/vial), red raspberry extract (*Rubus ideaus*) (1.10 g/vial), blueberry extract (*Vaccinium angustifolium*) (0.40 g/vial), cranberry extract (*Vaccinium macrocarpon*) (0.04 g/vial), Goji berries extract (*Licium barbarum*) (0.028 g/vial), pomegranate extract (*Punica granatum*) (0.006 g/vial), Green tea leave extract (*Camelia sinensis*) (0.005 g/vial)	2 vials/day (60 g)	Antioxidant properties
MonaVie	Acai berry juice 25%, concentrated fruit juice (apple, grape, pear, pineapple, cranberry, passion fruit, elderberry, prune, kiwi, blueberry, blackberry, wolfberry, cherry, pomegranate, count), fruit pulp (acerola , pear, banana, blueberry, black)	60-120 mL/day (60-120 g)	Antioxidant properties
Pau D'Arco	*Tabebula impetignos* powdered bark (500 mg/capsule)	4 capsules/day (2000 mg)	Antioxidant properites
PhytoC	Extract of: acerola, elderberry, lemon juice, wild rose	1 vial/day (30 g)	Strengthens natural resistance of organism.
Phytolife	Sodium – cooper chlorophyllin salt (22.32 mg/3 vials), *Mentha piperita* oil (48.12 mg/3 vials)	3 vials/day (22.5 g)	Antioxidant properties
PhytoMan	Extract of: aloe vera; fragrant cinnamon, wolfberry scarlet; turmeric long, multiflower; okra, chamomile, Chinese astragalus; ginseng	1 vial/day (30 g)	Increases fertility. Reduce the effects of andropause.
ReishiMax	*Reishi* mushroom extract (*Ganoderma lucidum*) (500 mg/capsule) with triterpenes (6%) and polysaccharides (13,5%)	2 capsules/day (1000 mg)	Stimulates immunity and resistivity of the organism

Table 1. Cont.

Dietary supplements/ products	Composition of the supplement/product	Recommended daily dose	Declared properties
SCleanUp	*Tamarindus indica L.* fruit extract (480 mg/vial), *Camellia sinensis L.* leave extract (120 mg/vial), *Cynara scolymus L.* extract (100 mg/vial), *Betula pendula Roth.* extract (100 mg/vial), *Filipendula ulmaria (L.) Maxim.* extract (50 mg/ vial), *Taraxacum officinale Weber* extract (50 mg/vial), *Foeniculum vulgare Miller* (50 mg/vial), *Arctium lappa L.* (25 mg/ vial), *Viola tricolor L.* (25 mg/ vial)	1 vial/day (12 g)	Digestive problems, detoxifies organism.
Super Digestion	Wine extract of: *Cynara scolymus* (450 mg/ vial), *Foeniculum vulgare* (225 mg/ vial), *Mentha piperita* (150 mg/ vial), *Melissa officinalis* (150 mg/ vial), *Angelica archangelica* (150 mg/ vial), *Carum carvi* (90 mg/vial), *Rosmarinus officinalis* (90 mg/ vial), *Taraxacum officinale* (75 mg/ vial), *Cinnamomum aromaticum Nees* (75 mg/ vial), *Gentiana lutea L.* (45 mg/ vial)	2 vials/day (30 g)	Digestive problems
Sweetacertabs	Powdered acerola fruit *(Malpighia punicifolia)* (244.8 mg/tablet), powdered blackcurrant fruit (23 mg/tablet) with Vitamin C (60 mg/tablet) natural flavor of raspberries (3.8 mg/tablet)	1 tablet/day (742 mg)	Antioxidant properites
Wilcashi Forte	*Vilcacora (Uncaria tomentosa)* shredded bark (100 mg/1 capsule), spores of the Reishi mushroom (*Ganoderma lucidum*m) (100 mg/L capsule)	4 capsules/day (800 mg)	Body health improvement. Stimulates immunity and resistivity of the organism
Wild rose juice	Wild rose (*Rosa canina*) extract	200 mL	Antioxidant properties
Xyliacertabs	Powdered acerola extract (240 mg/1 tablet), powdered blackcurrant (23 mg/1 tablet) with natural Vitamin C (60 mg/1 tablet), natural aroma of raspberries (4 mg/1 tablet)	1 tablet/day (742.5 mg)	Antioxidant properties

tion in µM, A – absorbance at 593 nm). Results were reported as mmol of Fe^{2+} per 1 g of supplement. All experiments were performed in triplicate.

***DPPH-EPR* assay**

For DPPH-EPR test, 50 or 100 µL of sample solution was mixed with 1 mL of acetone solution of DPPH (2.5 mM). After 2 min, the EPR spectra were recorded. The DPPH samples with demineralized water in place of a sample solution were used as intensity standards. The intensity was taken as the double integral of the spectra. Results were expressed as Trolox equivalents (µmol of TE per 1 g of the supplement) with the use of standard curve ($I_{EPR} = 5100\ c_{Tr} - 178.5$, where I_{EPR} – intensity of EPR signal, c_{Tr} – Trolox concentration in µmol/L). All experiments were performed in triplicate.

EPR measurements were performed on a EPR/SE/X (9.3 GHz) (Radiopan), with following parameters: central field 334 mT, sweep range 10 mT, sweep time 60 s, diode current 60%, attenuation 10 dB, modulation amplitude 0.2 mT, phase 90°.

Total polyphenols content determination

Polyphenols content was determined by modified Folin-Ciocalteu colorimetric method (16). Briefly, to 100 µL of Millipore water first 25 µL of sample, then 50 µL of working Folin-Ciocalteu reagent were added. After 3 min at room tempera-

ture, 120 µL of 20% sodium carbonate was added, and the reaction mixture was incubated for 30 min at 37°C. The absorbance at 765 nm was measured using microplate reader Synergy Mx (Biotec) and compared to a gallic acid calibration curve (C_{GA} = -30 + 187.2 A, where C_{GA} is gallic acid concentration in mg/L, A – absorbance at 765 nm). The results were expressed as gallic acid equivalents (GAE [mg/g]). All experiments were performed in triplicate.

Statistical analysis

All the results of antioxidant activity and antioxidants content determination (DPPH, ORAC, HORAC, FRAP and total phenolics) are presented as the mean ± SD. Statistical analyses were performed using STATISTICA (StatSoft Inc., USA). The number of variables (FRAP, ORAC, HORAC, DPPH-EPR, total phenolics) was reduced to two factors using factor analysis. The dietary supplements were described using two dimensional data (factor 1, factor 2) and grouped using Ward's hierarchical clustering method (agglomerative method).

RESULTS

The results of antioxidant activity determination by different methods and total polyphenol content of studied samples are presented in Table 2.

Using the ORAC assay, the hierarchy of antioxidant capacity ranging from 124 ± 20 to 21940 ± 420 µmol TE/g was obtained. The highest value was obtained for acerola extract, followed by Long Energy, and only for these samples were ORAC values similar to hydrophilic ORAC values obtained for dietary supplements based on extracts with well established antioxidant properties (12). Another eleven samples exhibited ORAC values over 1000 µmol TE/g (about half the ORAC average value of recommended five fruit and vegetable servings per day), which can be taken as a confirmation of their strong antioxidant properties (in order of decreasing values: ReishiMax, Vitamin C, Antiox, Immonobooster, Wilcashi Forte, Detox +, Duo Live (Day), Phyto C, Pau D' Arco, Duo Live (Night), Sweetacertabs).

The HORAC values ranged from 27.0 ± 6.3 µmol GAE/g for Duo Live (Day) to 2670 ± 510 mg GAE/g for Long Energy. The highest values were on the same level as those obtained by Anthony and Saleh (17) for sylimarin, which is also a dietary supplement with polyphenols as its main components. The majority of samples had HORAC results below 200 mg GAE/g, with only 12 of 29 samples that gave higher results (in decreasing order: Long Energy, ReishiMax, Wilcashi Forte, Antiox, Detox+, acerola extract, Pau D'Arco, Immonobooster, Gano Excel Cordyceps, CleanUp, wild rose juice, Duo Live (Night)). Among these 12 samples four had no declared antioxidant properties (ReishiMax, Wilcashi Forte, Gano Excel Cordyceps, CleanUp), but the producers declared immunostimulating or detoxifying action.

In FRAP assay the highest value was obtained for vitamin C (3602 ± 92 µmol Fe/g), and among supplements for Xyliacertabs (2468.8 ± 9.6 µmol Fe/g), and the lowest for PhytoMan (0.234 ± 0.028 µmol Fe/g). According to the results of this test, the supplements can be divided into two groups: the group with low activity (from 0.234 ± 0.028 to 81.74 ± 0.24 µmol Fe/g) and the group with high activity (from 307.0 ± 8.3 to 3602 ± 92 µmol Fe/g). However, the supplements with declared antioxidant activity were uniformly divided between both groups. On the other hand, among supplements without this declaration only the ReishiMax and Wilcashi Forte preparations had high FRAP value.

The best DPPH scavenger among studied supplements was acerola extract (535 ± 51 µmol TE/g), although its result was about 5 times lower than the result of vitamin C (2650 ± 140 µmol TE/g). The lowest value obtained in this test (10.90 ± 0.55 µmol TE/g for chinese yam) was about 25 times lower than the vitamin C result. It was the only antioxidant assay in which all studied supplements gave lower results than the standard antioxidant, i.e., vitamin C. Total polyphenols content of studied dietary supplements was in the range of 0.776-2255 µmol GAE /g. Similarly to the FRAP assay results, two groups of high and low polyphenol content could be seen (the low-polyphenol group with results in the range from 0.776 ± 0.041 to 44.4 ± 1.2 µmol GAE /g, with the lowest value for Floradix Ochrona Jelit, and the high-polyphenol group with results in the range from 115.0 ± 1.8 to 1198 ± 21 µmol GAE /g, with the highest value for acerola extract). Also in this case the high-polyphenol group is composed mainly of dietary supplements with declared antioxidant activity. It should be noted, however, that in the case of preparations containing vitamin C (as illustrated by the high value obtained for pure vitamin C (2255 ± 76 µmol GAE /g)) or reducing sugars the result of this test is influenced by these compounds.

Spearman's correlation coefficients between different antioxidant activity tests and total polyphenol content are shown in Table 3, and between different antioxidant activity tests results in Table 4. As can be seen, the strongest correlation was between

FRAP test results and polyphenol content, followed by ORAC test results and Folin-Ciocalteu assay. On the other hand, the HORAC test gave results that correlated the weakest with other antioxidant activity tests as well as with total polyphenol content.

In factor analysis, the first factor was due to FRAP, DPPH-EPR and Folin-Ciocalteu assays results, and explained 50% of the variability among studied samples, while the second factor was composed of ORAC and HORAC values and explained 29% of variability. The cluster analysis showed some grouping of the samples (Fig. 1a). As can be seen in Figure 1b, when data are grouped to two clusters, the average values for all assays for one cluster are higher than for the other cluster, i.e., both factors were responsible for this grouping. The majority of samples belonging to the first group were supplements with declared antioxidant properties. When the division of the samples into three clusters was done (Fig. 1c), one cluster consisted only of vitamin C (cluster 1). The other two clusters differed by the average values for all assays except

Table 2. Antioxidant activity and total polyphenol content (± mean standard deviation) of dietary supplements.

Supplement	ORAC [mmol TE/g]	HORAC [mmol GAE/g]	FRAP [mmol /g]	EPR [mmol TE/g]	Folin-Ciocalteu [µmol GAE /g]
Acerola extract, powder	21940 ± 420	510 ± 140	1270 ± 110	535 ± 51	1198 ± 21
ALFA AKTIV	730 ± 120	99 ± 20	405.0 ± 4.2	101.8 ± 5.1	115.0 ± 1.8
Antiox	3500 ± 130	706 ± 41	2250 ± 130	240 ± 12	798 ± 30
Chinese yam	320 ± 40*)	75.4 ± 7.7	15.86 ± 34	10.90 ± 0.55	14.519 ± 0.077
Detox +	2490 ± 160	690 ± 19	307.0 ± 8.3	67.3 ± 3.4	168.7 ± 7.1
Duo Live (Day)	1950 ± 480	27.0 ± 6.3	8.81 ± 0.53	168 ± 27	10.21 ± 0.38
Duo Live (Night)	1199 ± 92	217.4 ± 2.1	7.31 ± 0.35	140 ± 35	8.0 ± 0.5
Floradix Ochrona Jelit	380 ± 40	106 ± 17	16.5 ± 2.6	59 ± 3	12.70 ± 0.95
Floradix	401 ± 25	92 ±10	14.12 ± 0.17	340 ± 17	12.67 ± 0.32
Gano Excel Cordyceps	124 ± 20	335 ± 130	24.52 ± 0.13	29.9 ± 1.5	25.8 ± 1.8
Gano Excel Ganoderma	835 ± 67	131 ± 23	39.80 ± 0.11	92 ± 5	38.6 ± 2.2
Gano Excel Excellium	500 ± 51	93 ± 14	8.59 ± 0.11	86.5 ± 4.4	14.46 ± 0.71
Immunobooster	3490 ± 160	461 ± 62	1460 ± 130	108.9 ± 5.5	327.8 ± 5.9
Long Energy	10000 ± 1000	2670 ± 510	700 ± 100	105.4 ± 5.3	294.7 ± 7.7
Mistify	425 ± 12	137 ± 34	12.42 ± 0.18	30.8 ± 1.6	8.78 ± 0.36
MonaVie	284 ± 32	102.5 ± 3.5	22.34 ± 0.16	59 ± 3	7.28 ± 0.53
Pau D'Arco	1478 ± 83	505 ± 5	81.74 ± 0.24	31.6 ± 1.6	44.4 ± 1.2
Phyto C	1820 ± 320	136 ± 21	31.6 ± 4.0	320 ± 45	36.01 ± 0.79
Phytolife	391 ± 23	151 ± 31	4.066 ± 0.035	30.2 ± 1.6	2.23 ± 0.21
PhytoMan	683 ± 92	100 ± 21	0.234 ± 0.028	185 ± 22	0.776 ± 0.041
ReishiMax	5850 ± 410	2070 ± 270	336.6 ± 2.2	98 ± 5	218.6 ± 3.8
SCleanUp	947 ± 43	306 ± 17	54 ± 28	17.6 ± 0.9	38.0 ± 1.5
Super Digestion	498 ± 57	136 ± 25	28.4 ± 0.3	30.6 ± 1.6	13.81 ± 0.83
Sweetacertabs	1160 ± 150	116 ± 44	2210 ± 150	162.4 ± 8.2	381.5 ± 7.7
Wilcashi Forte	3350 ± 240	1230 ± 140	390 ± 4	41.7 ± 2.1	179.9 ± 8.3
Wild rose juice	448 ± 40	240 ± 40	52.1 ± 4.8	243 ± 45	43.4 ± 2.2
Xyliacertabs	829 ± 89	109.5 ± 5	2468.8 ± 9.6	152 ± 8	487.1 ± 5.9
Vitamin C	5400 ± 600	66.5 ± 4.4	3602 ± 92	2650 ± 140	2255 ± 76

Table 3. Spearman's correlation coefficients between different antioxidant activity tests and total polyphenol content.

Test	Spearman's correlation coefficient	Significance level
ORAC	0.69	0.00004
HORAC	0.38	0.04477
FRAP	0.96	< 0.00001
EPR	0.41	0.02553

Table 4. Spearman's correlation coefficients between antioxidant activity obtained in different assays.

Test	Spearman's correlation coefficient	Significance level
ORAC - HORAC	0.48	0.00780
ORAC - FRAP	0.62	0.00030
ORAC – EPR	0.49	0.00748
HORAC - FRAP	-	**0.06426**
HORAC - EPR	-	**0.45417**
FRAP - EPR	0.38	0.04491

diphenyl-1-picrylhydrazyl assay, though the difference of average result of polyphenol content determination was smaller than for ORAC and HORAC.

DISCUSSION

The ORAC and the HORAC assays, which are based on a hydrogen atom transfer (HAT) reaction and – in case of HORAC assay – also on the chelation of transition metals, an assay measuring electron transfer/reducing capacities, namely the ferric reducing antioxidant power (FRAP) and free radical scavenging properties by the diphenyl-1-picrylhydrazyl (DPPH) radical assays with the use of EPR spectroscopy, were used to investigate the antioxidant properties of hydrophilic fraction of 27 popular dietary supplements present on the Polish market, recommended for the general improvement of health and condition. Vitamin C was used as a standard, as a well-established antioxidant. It is also one of the antioxidants most abundant in the human diet and most popular as a dietary supplements component.

All of the studied dietary supplements exhibited some antioxidant properties, however, there was a large diversity among them. In all tests the values differed by orders of magnitude, with the biggest differences obtained in FRAP assay. Only two supplements gave high results in all antioxidant assays used as well as in Folin-Ciocalteu assay, namely acerola extract and Antiox preparation. This can be easily understood since acerola is an acknowledged rich source of vitamin C, it is also rich in phenolic compounds (18, 19). The declared composition of Antiox preparation also implies high antioxidants content, as it contains grape seed extract of well-established antioxidant properties (20) and is enriched with pure antioxidants (vitamin C, vitamin E and β-carotene).

Besides these two best antioxidant supplements, there were some preparations that gave high results only in some tests, among them Long Energy (high results in all tests except DPPH-EPR test, the highest HORAC value), ReishiMax (as Long Energy, high results in all tests except DPPH-EPR test, the second high HORAC value), Immunobooster (high ORAC, FRAP and total polyphenols content values, low HORAC and DPPH-EPR values) and Wilcashi Forte (high ORAC and HORAC, quite high FRAP value, low DPPH-EPR and polyphenol content). As can be seen, three out of four had high HORAC value. This was due probably to the presence of substantial amounts of substances other than polyphenols, as the correlation of HORAC with polyphenol content was the weakest among all antioxidant assays used. The possible candidates are polysaccharides from Reishi mushrooms extract (ReishiMax) or U. tomentosa bark and Reishi mushroom spores powder (Wilcashi Forte) or ginseng and

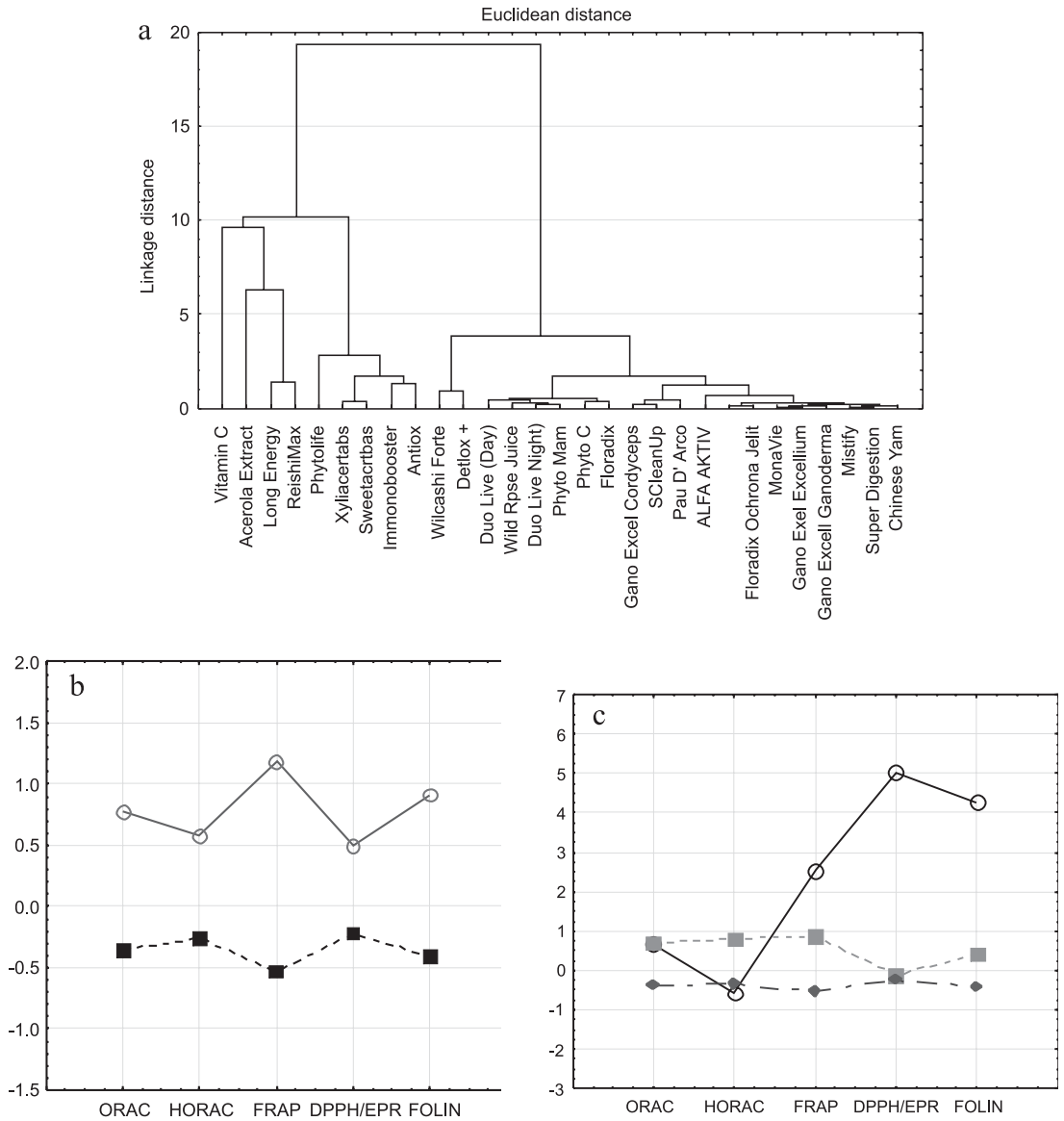

Figure 1. Hierarchical tree diagram (Ward's method) for dietary supplements with antioxidant properties (a), cluster profile plot for two clusters (standardized data) (b), cluster profile plot for three clusters (standardized data) (c)

Gingko biloba extract (Long Energy), which have chelating and antioxidant properties (21, 22), though their antioxidant activity towards DPPH radical is moderate. This would explain also the low values obtained in DPPH-EPR test. Also the fact that the only supplement with low HORAC value in this group was Immunobooster, which did not contain any source of polysaccharides, can be taken as the confirmation of this hypothesis.

Some of the supplements with antioxidant properties as a main or even the only declared action gave relatively low results in all antioxidant activity assays. The examples are AlfaAktiv, Mistify, wild rose juice, MonaVie. It should be noted, though, that these supplements are in the form of solutions, and their recommended daily dose is much higher in mass units than the daily dose of supplements in the form of tablets or capsules. Therefore, if this dose difference is taken into account, these liquid supplements can be treated as a source of antioxidants in the diet. The results of Ward's hierarchical clustering after recalculating the obtained results for the recommended daily dose of each supplement are shown in Figure 2 – different grouping than in the

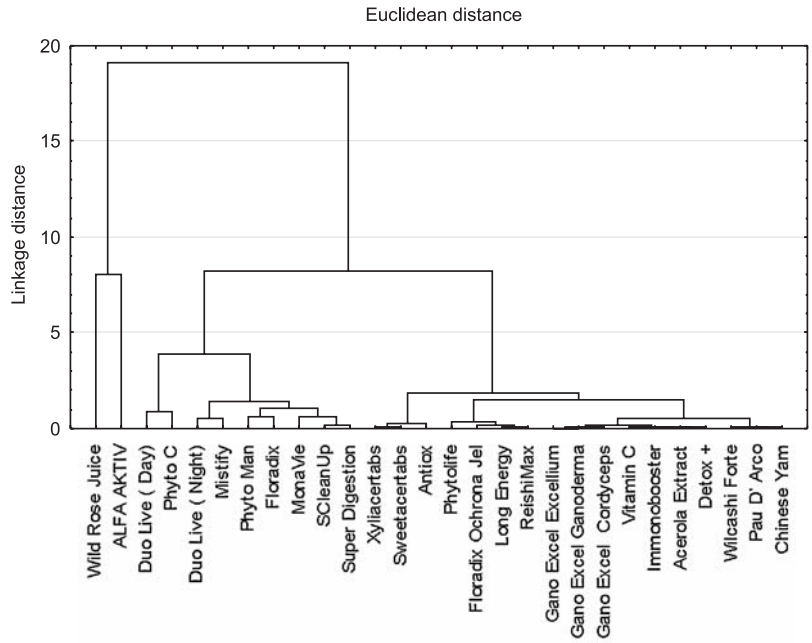

Figure 2. Hierarchical tree diagram (Ward's method) for dietary supplements with antioxidant properties after taking into consideration the daily dose

case of values taken for mass unit can be seen. Most notably, almost all supplements in the form of solution constitute the first three groups when the segregation into four clusters is done (the first two groups consisting of one preparation each), thus the serving form seems an important factor in analyzing antioxidant properties of food supplements and should be considered when choosing the best supplement.

The relatively weak correlations between different methods can be explained by different mechanisms underlying each method. Although ORAC and HORAC assays are both based on the HAT mechanism, in HORAC assay also the chelating properties of compounds such as polyphenols play an important role. Since the hydroxyl radicals are produced through a Fenton-like reaction, the metal complexation results in the prevention of their formation, not only their scavenging (14). Similarly, in the FRAP assay also the chelation of ferric/ferrous ions can influence the obtained results.

CONCLUSION

The determination of the antioxidant capacity of a series of dietary supplements available on the Polish market showed large diversity among the studied samples, confirming the need for devising control procedures for such preparations. However, the order of antioxidant activity of studied supplements depended on the method used. So, it should be stressed that it is important to run multiple antioxidant assays in order to get a better estimate of antioxidant capacity of dietary supplements, especially when comparing supplements with very diverse composition. It is also worth noting that when recommending a supplement with optimal antioxidant properties, the serving form should also be regarded.

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DRUG BIOCHEMISTRY

THE EFFECT OF IBUPROFEN ON bFGF, VEGF SECRETION AND CELL PROLIFERATION IN THE PRESENCE OF LPS IN HMEC-1 CELLS

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Abstract: Ibuprofen belongs to the group of non-selective cyclooxygenase (COX) inhibitors, also known as traditional non-steroidal anti-inflammatory drugs (NSAIDs). Bacterial lipopolysaccharide, an inflammatory mimicking agent, is responsible for the production of prostaglandins and growth factors (VEGF and bFGF), and as inflammation and angiogenesis are closely associated with osteoarthritis, these factors play a functional role in the cardiovascular system. Therefore, the main aim of our study was to examine the effect of ibuprofen on cell viability and proliferation of HMEC-1 cells and VEGF and bFGF secretion under the inflammatory conditions. The effect of NSAID and LPS on bFGF and VEGF was analyzed by ELISA. Cell viability was measured by the MTT method and the proliferation by the [^{3}H]-thymidine test. LPS at 100 μg/mL stimulated the secretion of VEGF and bFGF by HMEC-1 cells. Ibuprofen at concentrations of 0.1 and 1 mM intensified the secretion of LPS-induced VEGF in a statistically significant manner ($p < 0.05$). Both concentrations of ibuprofen inhibited LPS-stimulated bFGF secretion ($p < 0.05$) in HMEC-1 in a concentration-dependent manner. The non-selective COX inhibitor decreased proliferation and cell viability induced by LPS in a concentration-dependent manner. The observed effects of ibuprofen on endothelial cells may further explain its effects as well as other NSAIDs on the cardiovascular system function in cardiovascular diseases.

Keywords: bFGF, ibuprofen, LPS, VEGF, endothelial cells

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely-used drugs worldwide. They are used for their analgesic, antipyretic and anti-inflammatory effects, and their action is mediated by the inhibition of cyclooxygenase (COX) and prostaglandin production. Prostaglandins regulate vascular tone, blood coagulation as well as salt and water homeostasis in the mammalian kidney. Cyclooxygenase exists as two isoforms: COX-1, which is constitutive, and COX-2, which is induced by proinflammatory cytokines and endotoxin at inflammatory sites (1). Generally, NSAIDs are divided into two groups: one which comprises traditional, non-selective inhibitors of COX, such as ibuprofen, and another made up of selective COX-2 inhibitors (coxibes)(1, 2).

The endothelium forms a dynamic barrier between the vascular space and the tissues, and produces a variety of regulatory mediators such as nitric oxide, prostanoids, endothelins, angiotensin II, tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), von Willebrand factor (vWF), adhesion molecules, cytokines and growth factors. NSAIDs are known to have an influence on endothelial function, and may even induce endothelial dysfunction characterized by reduced vasodilation and increased endothelium-dependent contraction (3-8). Some data also indicate that endothelial dysfunction may be involved in the initiation of vascular inflammation and in the development of vascular remodelling. It is also an early determinant in the progression of atherosclerosis, and it is independently associated with increased risk for cardiovascular adverse events. The risk of cardiovascular complications associated with NSAIDs is currently broadly described (9). Although the mechanism behind the adverse cardiovascular effects of NSAIDs appears to be clear, the differences between the drugs themselves demand further analysis and a

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better understanding of the nature of their relationship with the endothelium.

Endothelial cells may also produce growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), which are responsible for generation of vasoactive such mediators as the prostaglandins PGI_2, PGE_2. Growth factors regulate proliferation of cells, inflammatory processes and neovascularization under physiological and pathological conditions (10, 11). It has been shown that exogenous bFGF increases angiogenesis and myocardial perfusion, promotes regeneration after myocardial infarction and thereby improves cardiac function (12, 13). From this point of view, it seems important to investigate the effect of ibuprofen, a widely used over-the-counter drug, on the secretion of endothelial growth factors under physiological conditions, and during inflammation. Therefore, the main aim of this study was to examine the effect of a non-selective COX inhibitor, ibuprofen, on cell viability, proliferation of HMEC-1 cells and VEGF and bFGF secretion under inflammatory conditions.

MATERIALS AND METHODS

Chemicals

MCDB 131 medium, fetal bovine serum, penicillin-streptomycin solution (5,000 units/mL penicillin and 5,000 µg/mL streptomycin sulfate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA (0.25% trypsin, 1 mM EDTA-4 Na) were purchased from Invitrogen (Carlsbad, USA). The cobalt chloride, thiazolyl blue tetrazolium bromide (MTT), human EGF, ibuprofen, lipopolysaccharides from *Salmonella enteritidis* (LPS) and hydrocortisone were purchased from Sigma Chemical Co. (St. Louis, USA).

Cell culture

HMEC-1 (human microvascular endothelial cells) were purchased from ATCC, catalog number ATCC-CRL-10636 (depositor Centers for Disease Control, Dr. Edwin W. Ades, Atlanta, USA). For experimentation, the cells between passages 10-31 were used. HMEC-1 cells were cultured in 25 cm^3 flasks in MCDB 131 medium supplemented with 10% fetal bovine serum, 10 ng/mL epidermal growth factor, 1 µg/mL hydrocortisone and penicillin-streptomycin solution, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were harvested every third day in a trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA). HMEC-1 cells were cultured according to the method described in the literature (14, 15) and the authors own modification.

ELISA assays

VEGF and bFGF concentrations in cell culture media were determined by commercially available ELISA kits according to the vendor's protocols (R&D System, Abingdon, UK).

MTT conversion

HMEC-1 cell viability was measured using the 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion method. Cells were seeded (50,000 cells/well) into 96-well plates. The treated cells were incubated for 24 h with 100 µg/mL LPS, 10 or 100 µM ibuprofen, LPS and ibuprofen or without tested chemicals (control group). All the substances were added at the same time. After incubation, 50 µL MTT (1 mg/mL, Sigma) was added and the plates were incubated at 37°C for 4 h. At the end of the experiment, the cells were exposed to 100 µL dimethyl sulfoxide, which enabled the release of the blue reaction product: formazan. The absorbance at 570 nm was read on a microplate reader and results were expressed as a percentage of the absorbance measured in control cells.

Assay of proliferative response of HMEC-1 cells

Cells were seeded in 96-well plates at a density of 50,000 cells per well in 100 µL of the culture medium and cultured for 1 day. On the day of the experiment, after rinsing the cells twice with PBS, fresh serum-free culture medium was added and the cells were incubated in the presence of drugs for 24 h at 37°C. Cell proliferation was determined by adding [^{3}H]-thymidine (0.5 µCi) 18 h before the end of incubation. The cultures were harvested with an automatic cell harvester (Scatron, Lier, Norway), and [^{3}H]-thymidine was estimated using a liquid scintillation counter MicroBetaTriLux (Perkin Elmer) (16).

Data analysis

All data are presented as the means ± SD (standard deviation). Statistical comparisons between the groups were performed using ANOVA and *post-hoc* comparisons were performed using the Student-Newman-Keuls test. The normal distribution of parameters was checked by means of the Shapiro-Wilk test. If the data were not normally distributed or the values of the variance (test F) were different, ANOVA with Kruskal-Wallis and Mann-Whitney's U test were used. All parameters were considered

significantly different if $p < 0.05$. The statistical data analysis was performed using Statgraphics 5.0 plus software.

RESULTS

The effect of ibuprofen on VEGF secretion under hypoxia and inflammatory conditions in HMEC-1 cells

It was found that 0.1 mM ibuprofen significantly increased VEGF level by 28%, and 1 mM by 113% (Fig. 1) in comparison with the control ($p < 0.05$). Ibuprofen at both concentrations also augmented secretion of VEGF in the presence of 100 µg/mL LPS and 200 µM $CoCl_2$. Non-selective COX inhibitor at 0.1 mM and 1 mM increased the secretion of VEGF by 5 and 7%, respectively, in comparison with $CoCl_2$ (200 µM). Application of ibuprofen (0.1 and 1 mM) with LPS (100 µg/mL) increased the level of VEGF by 8 and 71%, respectively. The observed effects were statistically significant ($p < 0.05$).

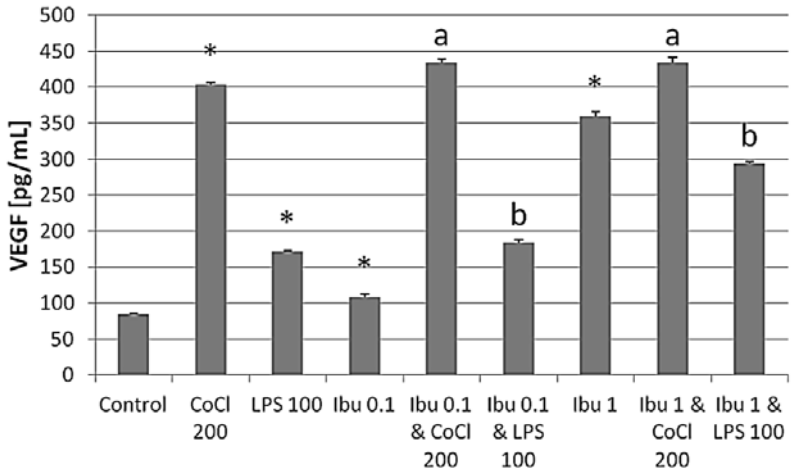

Figure 1. Effects of ibuprofen (0.1 and 1 mM) on VEGF levels in HMEC-1 cells in the presence of $CoCl_2$ (200 µM) or LPS (100 µg/mL). Bars represent the means (±SEM of 3-5 experiments). *$p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* $CoCl_2$ (200 µM); b – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: CoCl 200 - $CoCl_2$ 200 µM; LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

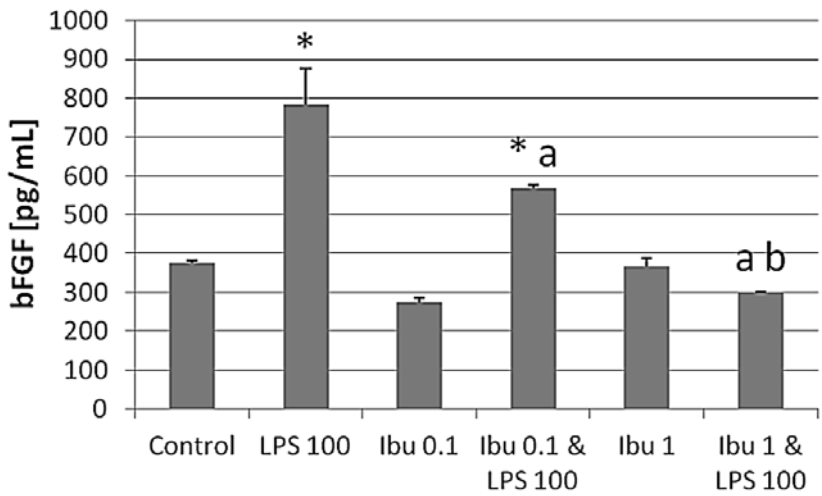

Figure 2. Effects of ibuprofen (0.1 and 1 mM) on bFGF levels in HMEC-1 cells in the presence of LPS (100 µg/mL). Bars represent the means (±SEM of 3-5 experiments). *$p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL); b – $p < 0.05$ *vs.* LPS (100 µg/mL) & ibuprofen (0.1 mM). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

The effect of ibuprofen on bFGF secretion under inflammatory conditions in HMEC-1 cells

Ibuprofen at concentrations of 0.1 and 1 mM had no effect on bFGF secretion (Fig. 2). The addition of 0.1 and 1 mM ibuprofen decreased the secretion of LPS-induced bFGF by 28 and 62%, respectively. The observed effects were statistically significant ($p < 0.05$). Simultaneously, 1 mM ibuprofen decreased the concentration of LPS-stimulated bFGF in cell culture media to a greater degree (by 48%) than 0.1 mM ibuprofen. This effect was also statistically significant ($p < 0.05$). Although 1 mM ibuprofen inhibited the secretion of bFGF in the presence of LPS in tested cells in comparison to control, this effect was not statistically significant.

The effect of ibuprofen on cell viability and proliferation in HMEC-1 cells

The next set of experiments analyzed cell viability based on MTT (Fig. 3), and proliferation by [^{3}H]-thymidine test (Fig. 4). LPS at a concentration of 100 µg/mL increased HMEC-1 cell proliferation by 8%, but only increased cell viability by 32%. Only LPS was found to have a statistically significant influ-

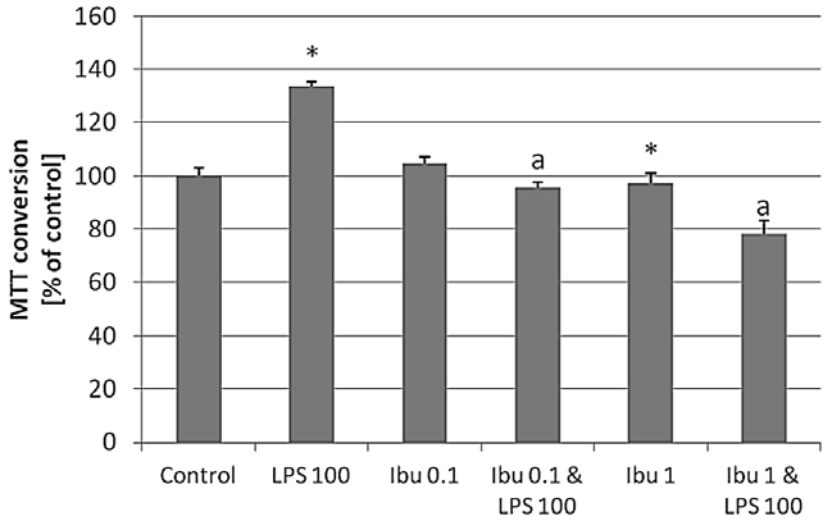

Figure 3. Effects of ibuprofen (0.1 and 1.0 mM) on cell viability of cultured HMEC-1 cells. The results are presented as a percentage in relation to the control value. Bars represent the means (±SEM of 4-15 experiments). * $p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

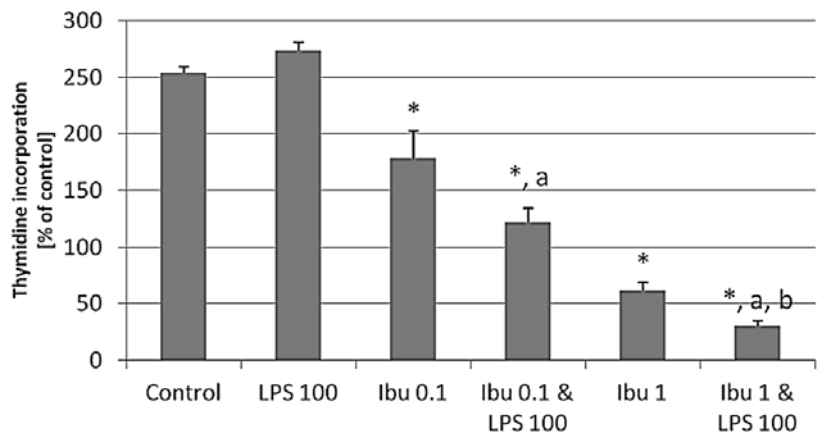

Figure 4. Effects of ibuprofen (0.1 and 1.0 mM) on thymidine incorporation in HMEC-1 cells. Bars represent the means (±SEM of 3-5 experiments). * $p < 0.05$ *vs.* control; a – $p < 0.05$ *vs.* LPS (100 µg/mL). Abbreviations used in this figure denote: LPS100 - LPS 100 µg/mL; Ibu 0.1 - ibuprofen 0.1 mM; Ibu 1 – ibuprofen 1 mM

ence on cell viability ($p < 0.05$). Ibuprofen did not affect cell viability at 0.1 mM, but decreased it in a statistically significant manner when used at 1 mM. Both concentrations (0.1 and 1 mM) of ibuprofen inhibited proliferation of cells by 76 and 30%, respectively ($p < 0.05$). Moreover, 0.1 and 1 mM ibuprofen inhibited LPS-induced proliferation by 52 and 89% and cell viability by 35 and 51%, respectively. These results were statistically significant ($p < 0.05$).

DISCUSSION

Our previous studies have shown that hypoxia (evoked by 3% O_2) increase the concentration of VEGF in human microvascular endothelial cells (HMEC-1), therefore in this study $CoCl_2$ is used inducing chemical hypoxia (15, 17-20) for comparison with LPS. In this study, bacterial lipopolysaccharide (LPS) is derived from *Salmonella enteritidis* cell walls and it causes the release of inflammatory cytokines. LPS is a less potent stimulator of VEGF secretion than hypoxia and, in contrast to hypoxia, stimulates the secretion of bFGF (17, 18, 20). The aim of this work was to determine the effect of ibuprofen, a non-selective COX inhibitor that inhibits the formation of prostanoids, on the secretion of VEGF under hypoxia and inflammatory conditions, as well as bFGF generation under inflammatory conditions.

Ibuprofen was found to induce production of VEGF and intensify the secretion of LPS-induced VEGF in a statistically significant manner ($p < 0.05$) (Fig. 1). The formation of VEGF is regulated by hypoxia and HIF (hypoxia induced factor) complex (19, 21, 22). NSAIDs have been observed to inhibit hypoxia-induced angiogenesis *via* increasing expression of the VHL tumor suppressor, reducing accumulation of HIF-1α, and consequently decreasing the secretion of VEGF in rat gastric microvascular endothelial cells, although non-selective COX inhibitor was not found to weaken the effect of LPS and $CoCl_2$ on VEGF secretion (21). These observations can be explained according to Palayoor et al. (23), who report that, when 2 mM ibuprofen is used in prostate cancer cells under hypoxic and normoxic conditions, a significantly higher concentration of ibuprofen is needed to inhibit the synthesis of HIF-1α and HIF-regulated gene products (VEGF) than prostaglandins (23). In the present study, ibuprofen used at the maximum concentration of 1 mM did not in fact inhibit VEGF secretion, but even potentiated it.

In a previous study, hypoxia was not seen to have an effect on bFGF level in HMEC-1 cells, although bFGF secretion was stimulated by LPS (20). Ibuprofen, used at both concentrations, inhibited the secretion of bFGF in the presence of LPS in tested cells to a statistically significant degree (Fig. 2.). Ibuprofen decreased level of LPS-induced bFGF in cell culture media to a greater degree when used at 1 mM than 0.1 mM ($p < 0.05$). Akarasereenont et al. demonstrated that LPS, the inflammatory mediator, is responsible for stimulation of COX and prostaglandin production in bovine aortic endothelial cells (BAEC) (24). Thus, the application of COX inhibitor reduces bFGF levels in HMEC-1 cells, which may indicate on participation of inflammation in the bFGF synthesis. The effect of ibuprofen on the secretion of bFGF may be reflected in the cardiovascular risk of NSAIDs, since bFGF (pro-angiogenic factor) is responsible for regeneration after myocardial infarction (11, 12).

According to the MTT and thymidine tests, the incubation of endothelial cells with 100 µg/mL LPS significantly stimulated cell survival (Fig. 3.) and proliferation (Fig. 4) ($p < 0.05$). Ibuprofen used at concentrations of 0.1 and 1 mM inhibited cell viability and the proliferative effect of 100 µg/mL LPS in comparison with the control and endothelial cells incubated with LPS. The cells exposed to LPS were found to release cytokines which activate COX-2. The products, prostaglandins, are responsible for endothelial cell proliferation (25, 26), COX inhibitors such as ibuprofen prevent their synthesis and hence, cell proliferation. Some studies have shown that sulindac and celecoxib inhibit the survival of endothelial cells and even induce their apoptosis (27, 28). Low concentrations of aspirin, which is used as an anti-aggregation drug, protect BAEC from apoptosis, while at relatively higher concentrations, when used as an anti-inflammatory, they induce apoptosis in endothelial cells (26). Taken together, the results imply that the inhibition of endothelial cell viability by high doses of NSAIDs lead to impaired endothelium function, which may have further consequences in terms of cardiovascular risk.

CONCLUSIONS

The obtained findings demonstrate that ibuprofen at concentrations of 0.1 and 1 mM both stimulates VEGF secretion and it increases bFGF reduction in concentration-dependent manner under inflammatory conditions. Ibuprofen decreases proliferation and cell viability induced by LPS in a concentration-dependent manner. The observed effects of ibuprofen on endothelial cells may further explain

its effects as well as other NSAIDs on the cardiovascular system function in cardiovascular diseases.

Acknowledgments

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ANTIPROLIFERATIVE EFFECT OF INOSITOL HEXAPHOSPHATE ON HUMAN SKIN MELANOMA CELLS *IN VITRO**

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Abstract: Human malignant melanoma is a highly metastatic tumor with poor prognosis. The majority of metastatic melanomas are resistant to diverse chemotherapeutic agents. Consequently, the search for novel antimelanoma agents continues. In recent years, the interest in plants and their biologically active constituents as a source of novel potential drugs significantly increased. Inositol hexaphosphate (IP6) is a naturally occurring compound that has been shown to inhibit the growth of a wide variety of tumor cells in multiple experimental model systems. The aim of this study was to evaluate the antiproliferative and cytotoxic influence of IP6 on melanotic melanoma cells *in vitro*. The A2058 cells used as a model of human skin *melanoma malignum* were exposed to different concentrations of IP6 (0.1-5 mM) for a various period of time and their growth was determined by sulforhodamine B assay after 24, 48 and 72 h. The cytotoxicity of IP6 was measured at 24 and 72 h by XTT assay. IP6 has been found to cause dose-dependent growth suppression of A2058 melanoma cells. At low concentrations (0.1 and 0.5 mM) it did not exert any influence on the cell proliferation as compared to control cultures. Higher concentrations of IP6 (from 1 to 5 mM) had a statistically significant, suppressive effect on cell proliferation after 24 h incubation. When the experimental time period was increased up to 72 h, statistically significant inhibition of cell proliferation was monitored at all IP6 concentrations used. Data obtained from XTT assay indicated that IP6 had dose- and time-dependent cytotoxic effect on melanoma cells. The results demonstrate the antiproliferative and cytotoxic properties of IP6 in a wide range of concentrations on human A2058 melanoma cells. Hence, it can be suggested that IP6 could have a promising therapeutic significance in treating cancer.

Keywords: inositol hexaphosphate, proliferation, A2058 cell line, *melanoma malignum*

Malignant melanoma (*melanoma malignum*) is one of the most lethal types of cancer and its rate of incidence has been rising for decades (1). It is an aggressive, highly metastatic tumor with poor prognosis arising from neoplastically transformed melanocytes. It is currently treated by local tumor surgery, radiotherapy and chemotherapy. The majority of metastatic melanomas are resistant to apoptosis and chemotherapeutic agents (2). Therefore, the development of a new, more effective therapy is justified.

Epidemiological studies suggest that diets rich in vegetables and fruits are associated with lower cancer risk (3). Much of the protective effect of these foods has been attributed to phytochemicals, which are naturally occurring, non-nutrient bioactive plant compounds that have disease preventive or therapeutic properties (4). In recent years, the interest in plants and their biologically active constituents as a source of novel potential anticancer drugs significantly increased. Among dietary agents inositol hexaphosphate (IP6), found in large amounts in cereal grain, nuts, legumes and oil seeds, has become a focus of interest in the latest years due to its chemopreventive properties against various types of cancer (5-7). Several studies documented the ability of IP6 not only to reduce the risk of developing cancer but also inhibit tumor progression in animal cancer models including colon, breast and prostate cancer (8). IP6 is supplied in daily human diet and rapidly absorbed from gastrointestinal tract (9). IP6 is also absorbed through the skin layers of humans and achieve important concentrations in tissues and biological fluids (10). *In vivo* studies have shown that IP6 is safe and devoid of toxicity (11).

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Little attention has been devoted, so far, to evaluation of the effects of IP6 on melanoma cells. Therefore, the aim of this study was to evaluate the antiproliferative and cytotoxic influence of IP6 in a wide range of concentrations on human melanotic melanoma A2058 cells *in vitro*.

EXPERIMENTAL

Cell culture

The human malignant melanoma cell line A2058 was obtained from LGC Promochem (Łomianki, Poland). The cells were routinely grown in

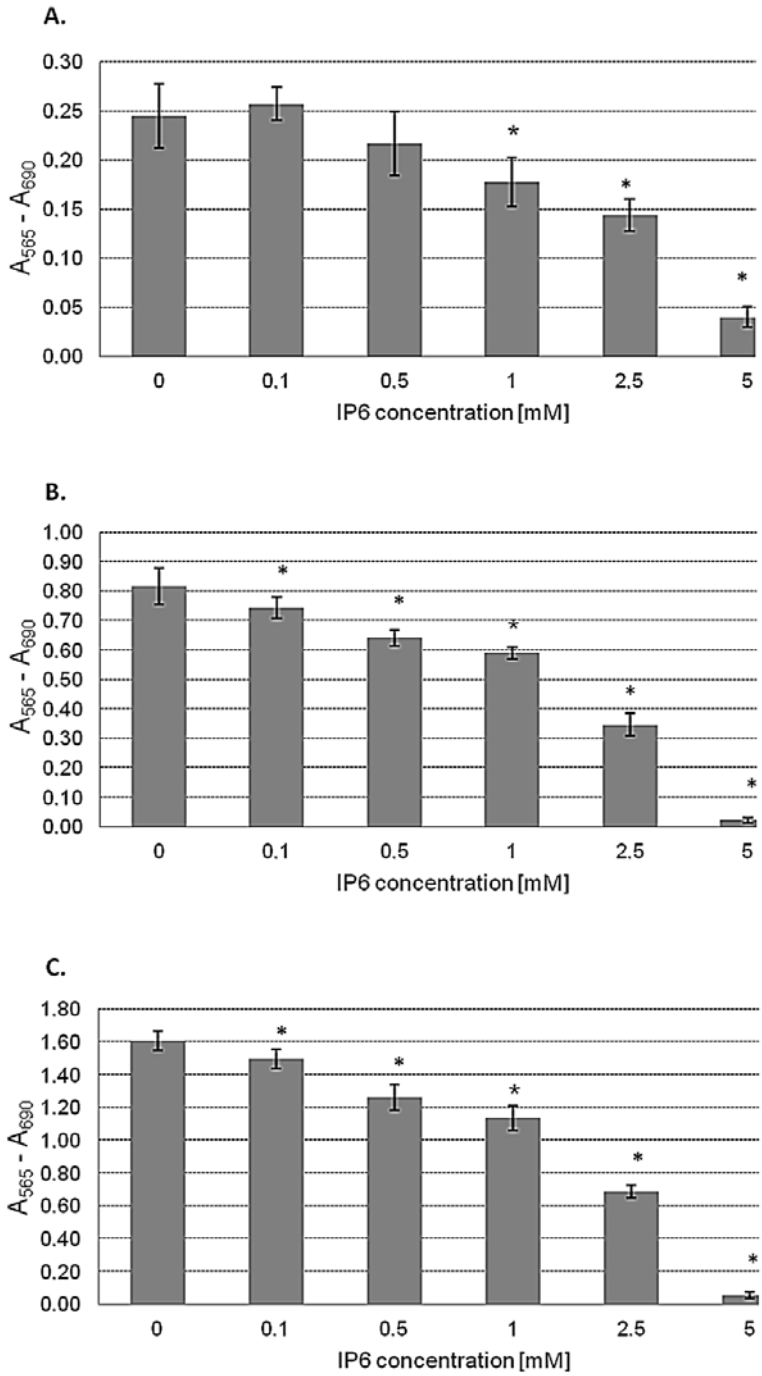

Figure 1. Growth of A2058 cells cultured in the presence of various concentrations of IP6 for 24 (A), 48 (B) and 72 (C) h. The results are expressed as the means ± SD; *$p < 0.05$ *vs.* control

RPMI 1640 medium (Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS; PAA), 100 U/mL penicillin (Sigma Aldrich), 100 µg/mL streptomycin (Sigma Aldrich) and 10 mM HEPES (Sigma Aldrich). Cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. When the confluence of cells reached approximately 80%, cells were trypsinized and transferred into another cultivation flask.

Cell proliferation assay

IP6 as dipotassium salt was purchased from Sigma Aldrich. Stock solutions of IP6 were prepared in distilled water and adjusted to pH 7.4. Before each experiment, stocks were diluted to the final concentration in culture medium. Cells were plated at initial density of 1×10^3 cell/well in 200 µL of culture medium in 96-well culture plates and allowed to adhere for 24 h. The media were than replaced with the fresh ones containing IP6 (0.1; 0.5; 1; 2.5 and 5 mM) and the cells were cultured for 24, 48 or 72 h. After removal of culture media from the wells, the cells were washed with phosphate-buffered saline (PBS) and fixed in 10% trichloroacetic acid. Proliferation of the cells was quantitated using *In Vitro* Toxicology Assay Kit, Sulforhodamine B Based (Sigma Aldrich) according to the manufacturer's protocol. Colorimetric results were measured using the MRX Revelation plate reader (Dynex Technologies) at $\lambda = 570$ nm and $\lambda = 690$ nm (reference wavelength).

XTT cytotoxicity assay

The cytotoxic effect of IP6 towards human melanoma A2058 cells was determined by *In Vitro* Toxicology Assay Kit XTT Based (Sigma Aldrich), which measures the metabolic activity of viable cells. This method is based on the ability of mitochondrial dehydrogenases of cells to cleave tetrazolium ring of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solution. A2058 cells were seeded at a number of 1×10^4 cells in 200 µL culture medium in 96-well cultured plates and allowed to attach and grow for 24 h. The medium was then replaced with 200 µL of fresh media containing desired concentrations of IP6 (0.1 – 5 mM) and incubated for the periods of 24 and 72 h. At the end of the treatment, the freshly prepared XTT reagent was added to each well as specified by the manufacturer. After adding the reactive solution to all of the wells, microplates were incubated for 2 h at standard conditions, and then, the absorbance was measured at 450 nm using a MRX Revelation plate reader (Dynex Technologies) with a reference wavelength of 690 nm. The cytotoxic effect of IP6 was expressed as a percentage of cell viability compared to untreated cells (12). The toxicity of IP6 was determined by means of the formula:

Cell viability (%) = Absorbance of treated cells/Absorbance of untreated cells × 100.

Statistical analysis

Statistical analysis was performed with the use of Statistica PL ver. 9.0 Software (StatSoft). All the results were expressed as the mean values ± standard deviation (SD). Student's *t*-test was used to assess statistical significance of difference between two groups. For multiple comparisons one-way analysis of variance (ANOVA) was performed followed by *post-hoc* Tukey's test. Differences with a probability (p) value less than 0.05 were considered statistically significant.

RESULTS

The influence of IP6 on cell proliferation

The A2588 cell line was used as a model of skin *melanoma malignum* cells. Cells were cultured in the presence of various concentrations of IP6 (0.1, 0.5, 1, 2.5 and 5 mM) for 24, 48 and 72 h (Fig. 1). The effect of IP6 on A2058 cell proliferation after 24 h is shown in Figure 1A. IP6 concentrations up to 0.5 mM did not inhibit the cell growth. A substantial growth inhibition was observed in cultures incubated with higher concentrations of IP6 (= 1 mM). The maximum decrease of cellular growth *versus* corresponding control cultures (84.36 ± 3.05%) was observed after treatment with the highest dose of IP6 (5 mM). The impact of IP6 on the A2058 growth was markedly enhanced when the experimental time period was elongated up to 72 h. IP6 at all concentrations used caused statistically significant inhibition of cell proliferation after both 48 h (Fig. 1B) and 72 h (Fig. 1C). The results indicate that IP6 reduced cellular growth in a concentration-dependent manner. Incubation with 5 mM IP6 resulted in almost complete (> 98%) reduction of A2058 cell growth.

Cytotoxic effect of IP6 on A2058 cells

The cytotoxic effect of IP6 on A2058 cells was determined after 24 and 72 h of treatment by XTT assay (Fig. 2). After 24 h of treatment with IP6, a significant decrease in mitochondrial dehydrogenases based cell viability was observed only in cultures exposed to the highest concentration of

IP6 (5 mM) (Fig. 2A). At cultures exposed to IP6 at concentrations up to 2.5 mM no significant alteration in cell viability was observed after 24 h (Fig. 2A). The experimental data presented in Figure 2 indicate that the exposure of melanotic cells to IP6 for 72 h caused more potent, dose-dependent cytotoxic effects (Fig. 2B). At low concentration (0.1 and 0.5 mM) IP6 caused statistically significant, about 12% decrease in activity of mitochondrial dehydrogenases in relation to control. This suppressive effect tends to be more enhanced with increasing IP6 concentration. The maximum reduction of cell viability (59%) was observed in cells treated with 5 mM IP6.

DISCUSSION

The continuous increase in incidence and failure of conventional therapies against the advanced malignant melanoma warrants development of novel effective therapeutics designed to target this malignancy. Thus, naturally occurring phytochemicals may be very beneficial in the prevention and treatment of skin cancers including melanoma due to their ability to modulate a plethora of molecular targets (4). The promising dietary component with chemopreventive and chemotherapeutic potential is IP6 (13). IP6 has received much attention for its role in cancer prevention and control of tumor growth, progression and metastasis. Anticancer effects of IP6 have been observed in various cancer models, both *in vitro* and *in vivo* (8, 11, 14, 15). Its anticancer actions involve boosting immunity, antioxidant properties, reducing cell proliferation and inducing apoptosis. Several intracellular signaling pathways including PI3K, MAPK, PKC, AP-1 and NFκB could be affected by IP6. IP6 also was shown to reverse the transformed phenotype of cells to the normal one, as demonstrated in colon, pancreatic and breast cancer cell lines (8, 16).

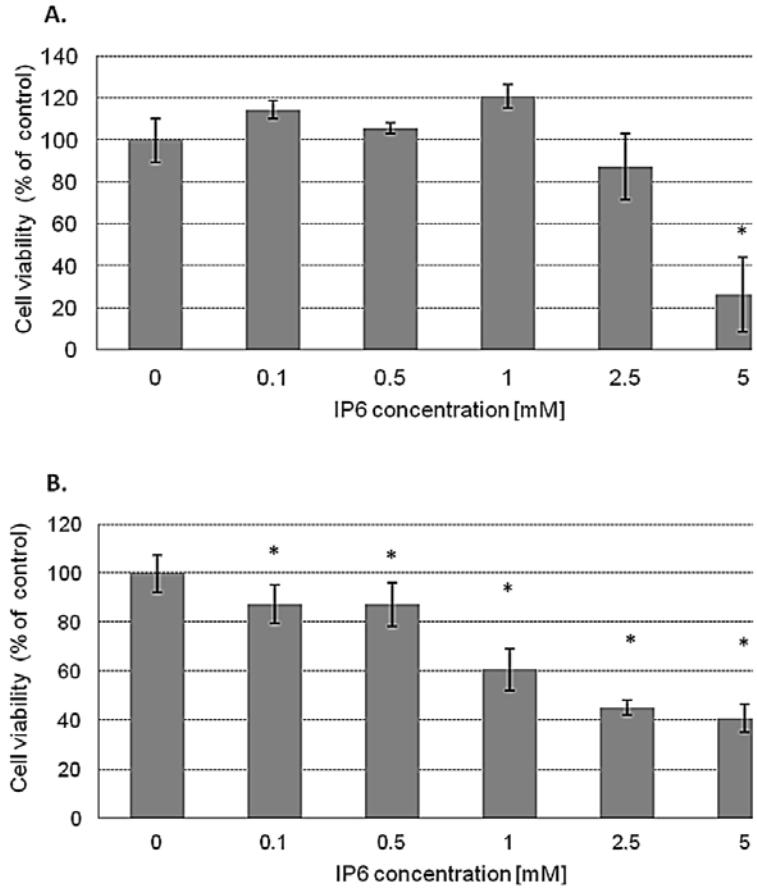

Figure 2. Cytotoxic effect of IP6 on human melanoma cells A2058 after 24 (A) and 72 (B) h. The results are expressed as the means ± SD; * - $p < 0.05$ statistically significant *vs.* control

One of the hallmarks of malignant cells is aggressive, uncontrolled proliferation. Numerous *in vitro* studies have demonstrated the ability of IP6 to inhibit growth of human leukemia, colon cancer, breast cancer, prostate cancer or hepatoma cells (11, 17). Cells of different origin presented different sensitivities to IP6 (8) and so, concentrations of IP6 capable of inhibiting cell proliferation varied depending on the cell line. For instance, 50% inhibition of growth of human rhabdomyosarcoma cells was achieved with IP6 at lower than 1 mM doses (18) and for human leukemia K-562 cells the IP6 concentration required to achieve IC_{50} (50% inhibition of cell number) was 7.5 mM (after 24 h) and 750 μM (after 120 h) (19).

Thus far, limited studies have investigated antiproliferative and antitumor effects of IP6 on skin cancer including melanoma. The prevention of skin cancer progression in a mouse model was supported by the observation of reduction in the number of DMBA-induced tumor formation following topical application of IP6 (20). Exposure to UVB radiation is believed to be responsible for most of the carcinogenic effects related to sun exposure and has been assumed to initiate mammalian melanoma (21, 22). IP6 exerted a protective effect against photoprecarcinogenesis as well as photocarcinogenesis. Recent studies indicated the effectiveness of oral administration of 2% IP6 in preventing preneoplastic or neoplastic skin lesions induced by UVB radiation in the SKH1 mouse model (23). Topical application of IP6 also decreased UVB-induced tumor incidence and multiplicity in mouse (24). Recent study showed that IP6 could prevent carcinogenesis by an impact on UVB-induced signal transduction pathways through inhibition of AP-1 and NFκB transcriptional activities (25).

Thus, in this study antiproliferative and cytotoxic potential of IP6 on human melanotic melanoma cell line A2058 was evaluated. The IP6 doses (0.1–5 mM) used in the experiment were based on earlier published studies, which in most cases used up to 5 mM IP6 concentration in cell culture treatments (26). The results of the present study show that exposure to IP6 decreases proliferation of A2058 melanoma cells in a concentration- and time-dependent manner. While IP6 in the doses up to 0.5 mM did not affect inhibition of cell growth after 24 h, a significant reduction in cell proliferative activity was manifested with higher concentrations of this compound. IP6 at all concentrations used caused statistically significant inhibition of cell proliferation when the experimental time period was elongated to 48 and 72 h. In this report, the cytotoxic property of IP6 on human melanoma cells has also been demonstrated. XTT assay showed dose- and time-related reduction of viability of A2058 cells with IP6 treatment and the observed effect enhanced with increasing IP6 concentration and prolongation of treatment time.

These data concur with the results published by Rizvi et al. (27), who demonstrated that IP6 (0.3-1 mM) inhibited growth of melanoma HTB68 cells in a dose-dependent manner after 72 h. Studies by Schneider et al. (28) showed that 1 mM IP6 significantly reduced the proliferation of MeWo cells derived from the lymphatic metastasis of a human cutaneous melanoma after 24, 48 and 72 h. Furthermore, treatment of MeWo cells with IP6 and pterostilbene, another phytochemical, produced a more profound extend of growth inhibition compared to treatment with either of them.

In conclusion, the present results show the antiproliferative and cytotoxic properties of IP6 against melanoma *in vitro*. Hence, it can be suggested that IP6 may have a promising therapeutic significance in treating melanoma.

Acknowledgment

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EFFECT OF CADMIUM(II) ON FREE RADICALS IN DOPA-MELANIN TESTED BY EPR SPECTROSCOPY*

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Abstract: Electron paramagnetic resonance (EPR) spectroscopy may be applied to examine interactions of melanin with metal ions and drugs. In this work EPR method was used to examination of changes in free radical system of DOPA-melanin - the model eumelanin after complexing with diamagnetic cadmium(II) ions. Cadmium(II) may affect free radicals in melanin and drugs binding by this polymer, so the knowledge of modification of properties and free radical concentration in melanin is important to pharmacy. The effect of cadmium(II) in different concentrations on free radicals in DOPA-melanin was determined. EPR spectra of DOPA-melanin, and DOPA-melanin complexes with cadmium(II) were measured by an X-band (9.3 GHz) EPR spectrometer produced by Radiopan (Poznań, Poland) and the Rapid Scan Unit from Jagmar (Kraków, Poland). The DOPA (3,4-dihydroxyphenylalanine) to metal ions molar ratios in the reaction mixtures were 2 : 1, 1 : 1, and 1 : 2. High concentrations of o-semiquinone (g ~2.0040) free radicals (~10^{21}-10^{22} spin/g) characterize DOPA-melanin and its complexes with cadmium(II). Formation of melanin complexes with cadmium(II) increase free radical concentration in DOPA-melanin. The highest free radical concentration was obtained for DOPA-melanin-cadmium(II) (1 : 1) complexes. Broad EPR lines with linewidths: 0.37-0.73 mT, were measured. Linewidths increase after binding of cadmium(II) to melanin. Changes of integral intensities and linewidths with increasing microwave power indicate the homogeneous broadening of EPR lines, independently on the metal ion concentration. Slow spin-lattice relaxation processes existed in all the tested samples, their EPR lines saturated at low microwave powers. Cadmium(II) causes fastening of spin-lattice relaxation processes in DOPA-melanin. The EPR results bring to light the effect of cadmium(II) on free radicals in melanin, and probably as the consequence on drug binding to eumelanin.

Keywords: DOPA-melanin, cadmium(II), DOPA-melanin-cadmium(II) complexes, free radicals, EPR spectroscopy

Free radical content in melanins is important for their ability of binding drugs to the chemical structure of these polymers (1-6). EPR studies show that in melanins exist o-semiquinone free radicals, and their concentrations depend on metal ions (1, 4-9) and drugs (4, 6, 8-11). Free radicals with unpaired electrons take part during formation of melanin-drug complexes (2, 4, 12). Free radical concentrations strongly depend on diamagnetic metal ions content in melanin polymers (4, 6, 13), so the knowledge about free radical systems in melanins is important to evaluate the binding ability of melanin to drugs. Our earlier EPR studies pointed out strong changes in free radical concentrations in *Cladosporium cladosporioides* melanin biopolymers (13-15). In this work, interactions of the model eumelanin – DOPA-melanin with cadmium(II) was examined by electron paramagnetic spectroscopy. Eumelanin generally exists in living organism (16-19), so it was the sample interesting for pharmacy. It was expected that interactions of eumelanin with cadmium(II) modify its ability to binding drugs.

The aim of this work is to examine the effect of cadmium(II) on properties and concentrations of free radicals in model eumelanin. The influence of different cadmium(II) concentrations on o-semiquinone free radicals in DOPA-melanin was tested. Magnetic interactions in DOPA-melanin complexes with cadmium(II) were compared with those in DOPA-melanin. Electron paramagnetic resonance spectroscopy was used as the method of direct studies of free radicals. EPR measurement do not change chemical structure of the tested samples, and the low amounts of the samples are needed (20-22).

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EXPERIMENTAL

Samples

DOPA-melanin and DOPA-melanin complexes with diamagnetic cadmium(II) ions of different concentrations were examined by EPR spectroscopy.

Synthetic DOPA-melanin – model eumelanin was obtained by autooxidative polymerization of 3,4-dihydroxyphenylalanine (L-DOPA, Sigma) in Tris-HCl buffer at pH 7.4 according to the Binns method (23).

Complexes of melanin samples with cadmium(II) ions were obtained as follows: 2.5, 5, and 10 mM $CdCl_2$ was added to 5 mM solution of L-DOPA in 50 mM Tris-HCl buffer at pH 7.4. The DOPA/cadmium(II) molar ratios in the reaction mixtures were 2 : 1, 1 : 1 and 1 : 2. Samples were incubated for 72 h at room temperature. Melanin sediments were centrifuged (2500 × g, 15 min) and then washed with deionized water.

EPR measurements

For free radicals in DOPA-melanin, and the complexes: DOPA-melanin-cadmium(II) (2 : 1), DOPA-melanin-cadmium(II) (1 : 1), and DOPA-melanin-cadmium(II) (1 : 2) EPR spectra were measured. The measurements were done by the use of an X-band (9.3 GHz) EPR spectrometer produced by Radiopan (Poznań, Poland). The modulation of magnetic field was 100 kHz. The Rapid Scan Unit from Jagmar (Kraków, Poland) was used for numerical detection of the lines. Microwave frequency was measured by MCM101 recorder of EPRAD (Poznań, Poland). The program LabVIEW 8.5 by National Instruments and the spectroscopic programs of Jagmar (Kraków, Poland) were applied in this work.

The following parameters of EPR spectra: g-values, linewidths (ΔB_{pp}) and integral intensities (I), were analyzed. Integral intensities depend on free radical concentration in the samples (20, 21). ΔB_{pp} changes with magnetic interactions in the samples and their values increase for the stronger dipolar interactions between free radicals (20-22). g-Value characterizes the type of free radicals. g-Values for free radicals were calculated from the resonance condition as (20): $g = h\nu/\mu_B B_r$, where: h - Planck constant; ν - microwave frequency; μ_B - Bohr magneton; B_r - induction of resonance magnetic field.

Free radical concentrations (N) in DOPA-melanin and DOPA-melanin-cadmium(II) complexes were determined by comparing the spectra of the analyzed sample with the spectra of references. The low microwave power of 2.2 mW was used to avoid microwave saturation effect. Free radical concentration is proportional to the integral intensity of EPR line, which is the area under the absorption curves (20-22). Integral intensities (I) were obtained by double integration of the first-derivative EPR spectra. The integral intensities of the EPR spectra of the tested DOPA-melanin and its complexes with cadmium(II) samples and for the reference – ultramarine (I_u) were compared. The second reference – a ruby crystal (Al_2O_3: Cr^3) was permanently placed in a resonance cavity. For each sample and for ultramarine the EPR line of a ruby crystal was measured. The concentration of the free radicals (N) in the tested samples was calculated according to the formula (20):

$$N = N_u[(W_u A_u)/I_u][I/(WAm)],$$

where: N_u - the number of paramagnetic center in the ultramarine reference; W, W_u - the receiver gains for the sample and ultramarine; A, A_u - the amplitudes of ruby signal for the sample and ultramarine; I, I_u - the integral intensities for the sample and ultramarine; m - the mass of the sample.

The influence of microwave power (M) in the range of 2.2-70 mW on the EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes was studied. The changes of integral intensities and ΔB_{pp} of EPR spectra with increasing of microwave power was determined.

Table 1. Free radicals concentrations (N), g-values, and linewidths (ΔB_{pp}) of EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. The data for EPR spectra recorded with microwave power of 2.2 mW.

Sample	$N \times 10^{21}$ [spin/g]	g ± 0.0002	ΔB_{pp} [mT] ± 0.02
DOPA-melanin	4.1	2.0040	0.37
DOPA-melanin-Cd(II) (2 : 1)	8.0	2.0042	0.48
DOPA-melanin-Cd(II) (1 : 1)	14.9	2.0041	0.73
DOPA-melanin-Cd(II) (1 : 2)	10.4	2.0041	0.57

RESULTS

EPR examination of DOPA-melanin and its complexes with diamagnetic cadmium(II) ions indicated their paramagnetic character. For all the tested melanin samples strong EPR spectra were measured. Binding of cadmium(II) by melanin changed its EPR line. EPR spectra of DOPA-melanin-complexes depend on cadmium(II) concentration in the samples. EPR spectra of DOPA-melanin and

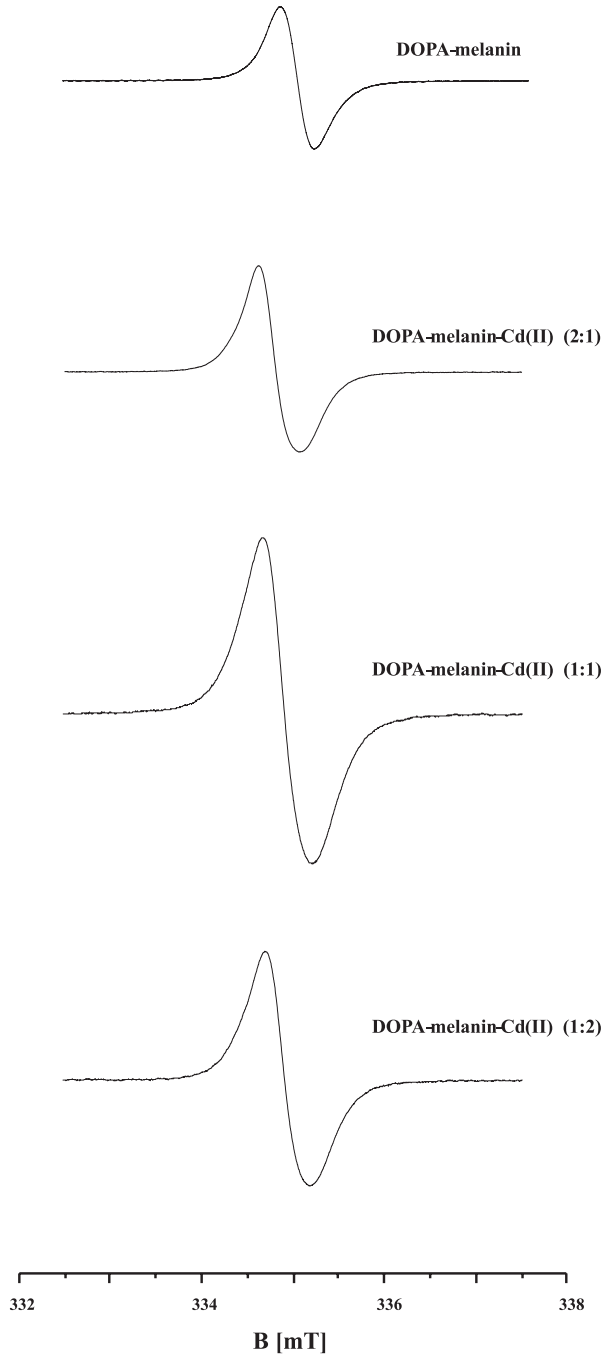

Figure 1. EPR spectra of DOPA-melanin, DOPA-melanin-cadmium(II) (2 : 1) complexes, DOPA-melanin-cadmium(II) (1 : 1) complexes, and DOPA-melanin-cadmium(II) (1 : 2) complexes. The EPR spectra were measured with microwave power of 2.2 mW. B - magnetic induction

DOPA-melanin-cadmium(II) complexes, for the DOPA/cadmium(II) molar ratios in the reaction mixtures amounts: 2 : 1, 1 : 1, and 1 : 2, are shown in Figure 1, respectively. EPR spectra of these samples are single asymmetric lines. The parameters of the measured EPR spectra recorded with microwave power of 2.2 mW and free radical concentrations in DOPA-melanin and the complexes DOPA-melanin with cadmium(II) are presented in Table 1.

EPR spectra of the samples are broad with ΔB_{pp} in the range from 0.37 to 0.73 mT (Table 1). Dipolar interactions are responsible for line broadening of the recorded EPR spectra. EPR lines of DOPA-melanin complexes with cadmium(II) are broader (ΔB_{pp}: 0.48-0.73 mT) than EPR lines of DOPA-melanin (ΔB_{pp}: 0.37 mT) (Table 1). The highest linewidth (ΔB_{pp}: 0.73 mT) characterizes EPR spectrum of DOPA-melanin-cadmium(II) (1 : 1) complexes. The relatively lower linewidths were obtained for DOPA-melanin-cadmium(II) complexes (2 : 1) (ΔB_{pp}: 0.48 mT) and DOPA-melanin-cadmium(II) complexes (1 :2) (ΔB_{pp}: 0.57 mT).

g-Values in the range of 2.0040-2.0042 (Table 1) point out that o-semiquinone free radicals exist in DOPA-melanin and DOPA-melanin-cadmium(II) complexes.

The high values (~10^{21}-10^{22} spin/g) of o-semiquinone free radical concentrations for DOPA-melanin and DOPA-melanin-cadmium(II) complexes were measured (Table 1). Addition of cadmium(II) to melanin polymer causes an increase of free radical concentration. Free radical concentrations in DOPA-melanin and DOPA-melanin-cadmium(II) (2 : 1) complexes are equal ~10^{21} spin/g, while free radical concentrations in DOPA-melanin-cadmium(II) (1 : 1) and DOPA-melanin-cadmium(II) (1 : 2) complexes are ~10^{22} spin/g. Free radical concentration in DOPA-melanin complexes with cadmium(II) increases as follows: DOPA-melanin-cadmium(II) (2 : 1) < DOPA-melanin-cadmium(II) (1 : 2) < DOPA-melanin-cadmium(II) (1 : 1) (Table 1).

Magnetic interactions in melanin samples were tested by the continuous microwave saturation of their EPR spectra. The parameters of the spectra depend on microwave power. The effect of microwave power on linewidth and integral intensity of the EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes are presented in Figures 2 and 3, respectively.

The ΔB_{pp} values increase with increasing microwave power both for DOPA-melanin and DOPA-melanin-cadmium(II) complexes, independ-

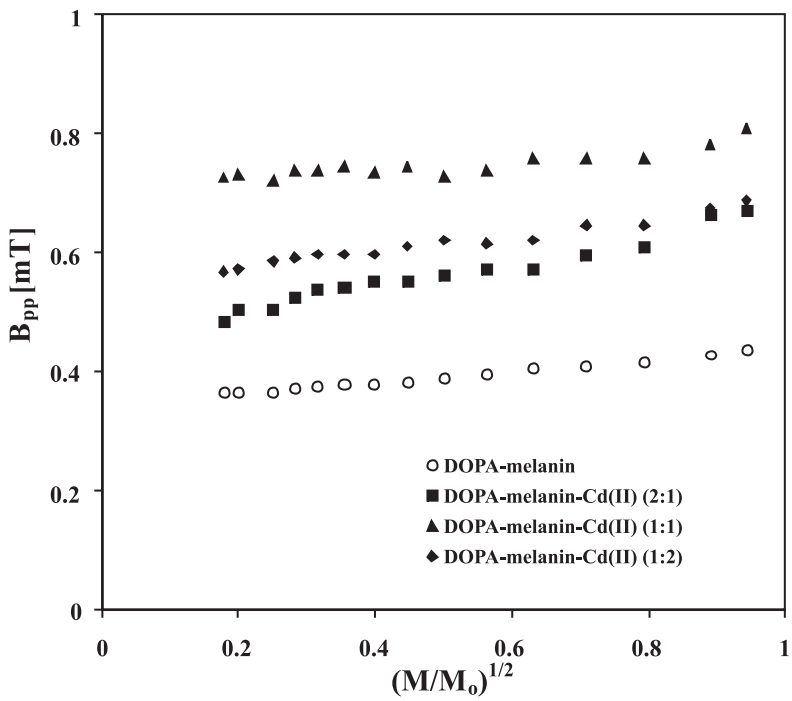

Figure 2. Influence of microwave power (M/M_o) on linewidth (ΔB_{pp}) of EPR spectra of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. M, M_o - the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

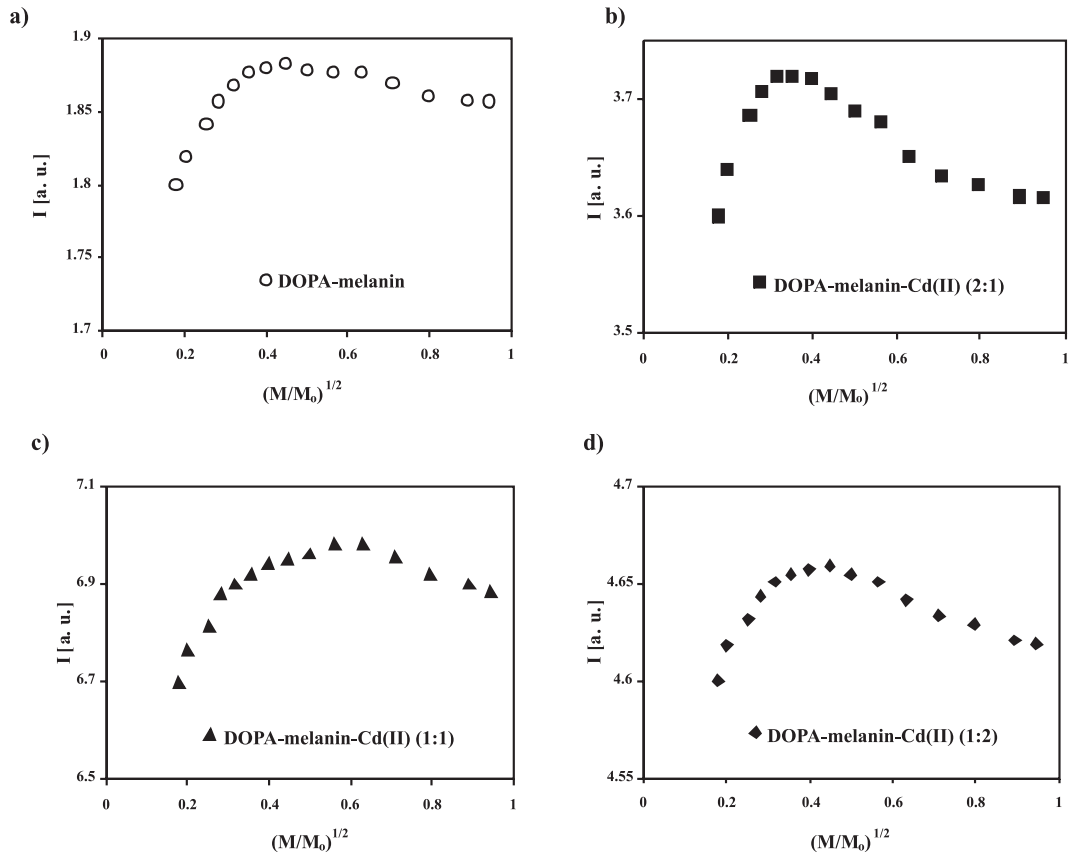

Figure 3. Influence of microwave power (M/M_o) on integral intensity (I) of EPR spectra of DOPA-melanin (a), DOPA-melanin-cadmium(II) (2 : 1) complexes (b), DOPA-melanin-cadmium(II) (1 : 1) complexes (c) and DOPA-melanin-cadmium(II) (1 : 2) complexes (d). M, M_o - the microwave power used during the measurement of the spectrum and the total microwave power produced by klystron (70 mW), respectively

ent on cadmium amount in melanin polymer (Fig. 2). Integral intensities increase with increasing microwave power, reach the maximum value, and after they decrease (Fig. 3). Such correlations between microwave power and both linewidths (Fig. 2) and integral intensities (Fig. 3) point out that the EPR lines of DOPA-melanin and DOPA-melanin-cadmium(II) complexes are homogeneously broadened.

The EPR lines of all the examined melanin samples are saturated at low microwave powers (Fig. 3), so slow spin-lattice relaxation processes exist in DOPA-melanin and DOPA-melanin complexes with cadmium(II). Cadmium(II) strongly affect spin-lattice relaxation processes in DOPA-melanin and change the values of microwave power of saturation. Integral intensities of EPR lines of DOPA-melanin-cadmium(II) complexes begin decrease with microwave power for its higher values (Fig. 3). The spin-relaxation processes in DOPA-melanin-cadmium(II) complexes (Fig. 3b-d) are relatively faster than in DOPA-melanin (Fig. 3a).

DISCUSSION AND CONCLUSIONS

The performed EPR examination of melanin samples are very important for drug binding to melanin polymer in the presence of cadmium(II). It is known that o-semiquinone free radicals in DOPA-melanin take a part in binding of drugs to its structure (1-11). Our studies point out an application of EPR spectroscopy to determine the type of free radicals in melanin complexes and their concentration in the samples, and to characterize magnetic interactions.

Diamagnetic cadmium(II) increases the free radical concentrations in DOPA-melanin (Table 1).

The high content of o-semiquinone free radical concentrations in DOPA-melanin-cadmium(II) complexes points out that probably the strong possibilities of binding of drugs *via* these paramagnetic centers exist. It is expected that participation of o-semiquinone free radicals in binding of drugs will increase similarly to an increase of free radical concentrations in melanin: DOPA-melanin-cadmium(II) (2 : 1) < DOPA-melanin-cadmium(II) (1 : 2) < DOPA-melanin-cadmium(II) (1 : 1) (Table 1). The lower free radical concentration in DOPA-melanin-cadmium(II) (1 : 2) (Table 1) is caused by recombination of free radicals during synthesis.

The increase of free radical concentration in melanin samples after complexing with cadmium(II) was observed by us earlier (15). Free radical concentration in DOPA-melanin complexes with cadmium(II) was ~10^{19} spin/g (15) and this value was lower than for DOPA-melanin complexes with cadmium(II) tested in this work (~10^{21}-10^{22} spin/g; Table 1). Differences between values of free radical concentrations result from different methods of obtaining the melanin samples. Complexes of cadmium(II) with DOPA-melanin in this work were formed during synthesis of this polymer ($CdCl_2$ was added to solution of L-DOPA in Tris-HCl buffer), whereas in previous work, cadmium(II) was added to DOPA-melanin after process of its synthesis.

The increase of free radical concentration in melanin after complexing with cadmium(II) may be responsible for major chemical reactions, because of chemical activity of free radicals containing unpaired electrons. Free radicals may interact with oxygen molecules in the melanin environment or with free radicals in others structures. Such reactions should be tested in the future.

In this work the effect of microwave power (2.2-70 mW) on the parameters of EPR lines of DOPA-melanin and its complexes with cadmium(II) (Figs. 2, 3) was compared. According to the theory of electron paramagnetic resonance, the effect of microwave power (M) on integral intensities and ΔB_{pp} of the EPR spectra depend on free radicals distribution (homogeneous or non-homogeneous) in chemical structure of the samples (20, 21). For homogeneous broadened EPR lines the integral intensity increases with increasing of microwave power and for the higher microwave powers its value decreases as the result of microwave saturation. The increase of ΔB_{pp} with increasing of microwave power is characteristic for the homogeneously broadened EPR lines. For non-homogeneous broadening of EPR lines, the integral intensity increases with increasing of microwave power (M), but for the higher microwave powers its value does not change. ΔB_{pp} of the non-homogeneously broadened EPR lines is constant and they do not depend on microwave power. Correlations presented in Figures 2 and 3 prove that the EPR lines of DOPA-melanin and DOPA-melanin complexes with cadmium(II) are homogeneously broadened. Homogeneous broadening of EPR lines of melanin was observed by us earlier (6, 7, 9, 10, 12, 13). Homogenous broadened EPR lines characterize DOPA-melanin-complexes with zinc(II) (6, 13), copper(II) (5, 6, 8, 9, 12, 13), and iron(III) (7). Homogeneously broadened EPR lines were measured for melanin complexes with netilmicin (6, 8), kanamycin (9, 12), and moxifloxacin, ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin (10, 11). Cadmium(II) changes magnetic interactions in DOPA-melanin. The broader EPR lines of DOPA-melanin-cadmium(II) complexes relative to DOPA-melanin (Table 1) indicate stronger dipolar interactions in DOPA-melanin after addition of cadmium(II). This effect is the highest for DOPA-melanin-cadmium(II) (1 : 1) complexes. Dipolar interactions increase with decreasing the distances between unpaired electrons of free radicals (20), so the short distances between o-semiquinone free radicals exist in DOPA-melanin-cadmium(II) (1 : 1) complexes. Dipolar interactions and distances between free radicals are relatively lower in DOPA-melanin-cadmium(II) (1 : 2) and DOPA-melanin-cadmium(II) (2 : 1) complexes with relatively narrower EPR lines (Table 1).

The changes of integral intensities (I) of EPR lines with microwave power (Fig. 3) are characteristic for slow spin-lattice relaxation processes. The slow and fast spin-lattice relaxation processes in the samples differ in microwave saturation of EPR lines (20). The higher power of microwave saturation of EPR lines reveals the samples with faster spin-lattice relaxation processes than the samples with the slow spin-lattice relaxation processes (20-22). Cadmium(II) in melanin causes fastening of spin-lattice relaxation processes, because the shift of microwave saturation of EPR lines to the higher values of microwave power is observed (Fig. 3b-d).

The several important conclusions for pharmacy may be drawn from our EPR studies of DOPA-melanin and DOPA-melanin-cadmium(II) complexes. Cadmium(II) increases free radical concentration in DOPA-melanin, so the binding ability of drugs to melanin *via* free radicals rises. This ability is the highest for DOPA-melanin-cadmium(II) (1 : 1) complexes, because of the highest o-semiquinone free radical concentration observed in these com-

plexes. The relatively lower binding of drugs is expected for DOPA-melanin-cadmium(II) (1 : 2) complexes and DOPA-melanin-cadmium(II) (2 : 1) complexes with the lower free radical concentration compared to DOPA-melanin-cadmium(II) (1 : 1) complexes. The influence of cadmium(II) on the ability of melanin to binding drugs is only suggested by us, and it should be tested in the next works. Our suggestion results from our earlier EPR studies of melanin complexes with drugs: netilmicin, kanamycin, moxifloxacin, ciprofloxacin, lomefloxacin, norfloxacin and sparfloxacin (6, 8-12), where free radical contents in melanin changed after introducing of drug molecules to this polymer.

EPR examination broadens the knowledge about free radicals in DOPA-melanin complexes with cadmium(II) of different concentrations of these diamagnetic ions. All the tested complexes are strongly paramagnetic and the formation of o-semiquinone free radicals dominates in DOPA-melanin-cadmium(II) (2 : 1) and DOPA-melanin-cadmium(II) (1 : 1) complexes. Recombination of free radicals appears during complexation of DOPA-melanin with cadmium(II) for DOPA-melanin-cadmium(II) (1 : 2) complexes. Strong dipolar interactions and slow spin-lattice relaxation processes exist in all the tested melanin samples. Cadmium(II) changes both dipolar interactions and spin-lattice relaxation processes in DOPA-melanin. Dipolar interactions increase in DOPA-melanin complexes with cadmium(II) and faster spin-lattice processes appear, relatively to DOPA-melanin. EPR spectroscopy with microwave from the X-band (9.3 GHz) is the useful method to examine free radicals in melanin complexes with cadmium(II) ions. The fine example of application of EPR method in pharmacy was presented in this work.

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MOLECULAR EFFECTS OF AMINE DERIVATIVES OF PHENOTHIAZINE ON CANCER CELLS C-32 AND SNB-19 *IN VITRO**

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Abstract: Cancer therapy is challenging for scientists because of low effectiveness of so far existing therapies (especially in case of great invasiveness and advanced tumor stage). Such need for new drug development and search for more efficient new findings in therapeutical applications is therefore still valid. There are also conducted studies on modifying so far existing drugs and their new methods of usage in oncology practice. One of them is phenothiazine and its derivatives which are used in psychiatric treatment for years. They also exhibit antiprion, antiviral, antibacterial and antiprotozoal properties. Cytotoxic activity, influence on proliferation, ability to induce apoptosis suggest also a possibility of phenothiazine derivatives usage in cancer cells termination. The aim of our the study was to evaluate the influence of two amine derivatives of phenothiazine on cancer cells *in vitro*. Amelanotic melanoma C-32 cell line (ATCC) and glioma SNB-19 cells (DSMZ) were used in this study and two derivatives were analyzed. In view of examined substances tumor potential toxicity cells proliferation and viability exposed to phenothiazine derivatives were established. Cell cycle regulatory genes expression (*TP53* and *CDKN1A*), S-phase marker - H3 gene and intracellular apoptosis pathway genes (*BAX, BCL-2*) were analyzed using RT-QPCR method. The influence of examined derivatives on total cell oxidative status (TOS), total antioxidative status (TAS), malondialdehyde concentration (MDA) and superoxide dismutase activity (SOD) were analyzed. As a result, examined phenothiazine derivatives cytotoxic action on C-32 and SNB-19 and also cells proliferation inhibition were determined. Cell cycle regulatory genes (*TP53, CDKN1A*) expression and protein products of genes involved in mitochondial apoptosis pathway (*BAX, BCL-2*) expression are changed by the presence of phenothiazine derivatives during culturing. There were also noted small changes in redox potential in cells exposed to two mentioned phenothiazine derivatives.

Keywords: phenothiazine derivatives, C-32, SNB-19

Abbreviations: *BAX* - pro-apoptotic BAX protein gene, *BCL-2* - anti-apoptotic BCL-2 protein gene, *CDKN1A* - cyclin-dependent kinase inhibitor 1 (or CDK-interacting protein 1) gene, *H3* - H3 histone gene (S-phase marker, proliferation indicator), MDA - malondialdehyde, Real-Time RT-QPCR - Real Time Reverse Transcription Quantitative Polymerase Chain Reaction, SOD - superoxide dismutase activity,TOS - total oxidative status, *TP53* - gene of the cell cycle regulatory P53 protein

Cancer is still very significant therapeutical problem. Rising number of oncology patients leads scientists and doctors to search for new therapeutical options including great interest in looking for new drugs and substances with antiproliferative and cytotoxic actions directed to tumor cells. Difficulties in this field derives from tumor cells themselves. These are resistant to apoptosis, often indicate multi-drug resistance and lowered oxygen demand. Additionally, impeded drug delivery system in

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tumors caused by incorrect vascular construction, interstitial pressure and pH changes is observed. Because of mentioned factors and problems, new anticancer drugs are sought. These substances are often modified existing anticancer drugs with changed chemical structure and mode of action.

Great interest in cancer treatment is laid in phenothiazines and their derivatives. This group includes aliphatic, piperazine, piperidine trycyclic compounds, where two benzene rings are bound by sulfur and nitrogen atoms. Phenothiazines exhibit an antipsychotic-neuroleptic actions, antihistamine, antitussive and antimetic actions, also antiprion, antiviral, antibacterial and antiprotozoal activity (1-3). It was also observed that patients treated with phenothiazines less likely suffer from cancer (4). Some of phenothiazine derivatives indicate preventive antitumor actions (their biological anticancerogenous activity) by viruses destruction (5-9). Moreover, phenothiazines can indicate antiproliferative activities by calmodulin antagonists activity, which binds calcium ions and takes part in cells proliferation induction (10). Phenothiazines can also act as both pro- and antioxidants (11, 12). It was indicated that some of these derivatives inhibit transporting functions of P-glycoprotein, causing better ability to penetrate directly by cytostatics into tumor cells and affect them (2, 13). They may also have pro-apoptotic properties of deciding on the intracellular ceramide content, which stimulates accession to apoptosis (14). It was proved that phenothiazine derivatives actions and effectiveness depend on kind and location of substituent in the particular compound chemical structure (2, 8, 15, 16). For example, -CF_3 substituent in phenothiazine derivatives in C2 phenothiazine ring position is characterized as highly proapoptotic while substances with -Cl atom in the same C2 position act much less proapoptotic (fluphenazine is classified as highly proapoptotic drug and chlorpromazine is classified as slightly proapoptotic compound). Nowadays, research are focused on new derivatives with higher proapoptotic activity and with precisely directed antitumor actions (17).

The aim of the present study was to examine two amine derivatives of phenothiazine impact on tumor cells in cell culture conditions.

EXPERIMENTAL

Cell culture

In the study, amelanotic melanoma C-32 (ATCC, USA) and glioma SNB-19 (DSMZ, Germany) cells were analyzed. Cells were cultured by using DMEM medium (Lonza, Switzerland) with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and penicillin (1000 units/mL) with streptomycin (10 mg/mL) mixture (Biological Industries, Israel).

Phenothiazine derivatives

Derivatives of phenothiazine used were: A) 9-(*N*-piperidyl)-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride and B) 9-amino-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride (Fig. 1). They were synthesized in the Department of Organic Chemistry (Medical University of Silesia in Katowice, School of Pharmacy with the Division of Laboratory Medicine).

Cytotoxicity

Cells viability was analyzed by C-32 and SNB-19 culturing in chosen phenothiazine derivatives concentrations (0.1, 0.5, 1, 5, 10, 50 and 100 µg/mL). The number of cells in cultures was evaluated after 72 h of incubation with the test derivatives in media without the addition of FBS. WST-1 test (Roche Diagnostics GmbH, Germany) was used to examine viability cells number and LDH (Roche Diagnostics GmbH, Germany) test was used to examine dead cells number. Absorbance analyses were made by using UVM340 microplate reader (Biogenet, Polska).

Proliferative activity

The influence of phenothiazine derivatives on cells proliferative activity was evaluated after cells incubation by 72 h with examined compounds in media with FBS (Biological Industries, Israel) con-

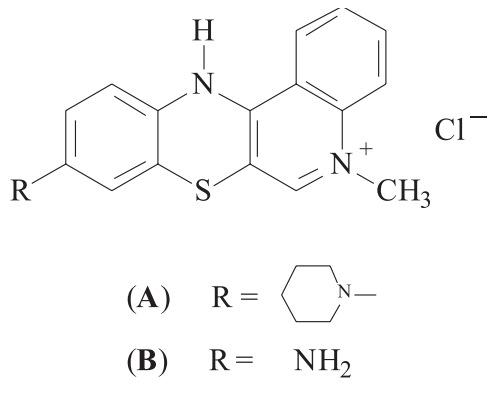

Figure 1. Derivatives of phenothiazine tested. A - 9-(*N*-piperidyl)-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride; B - 9-amino-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride

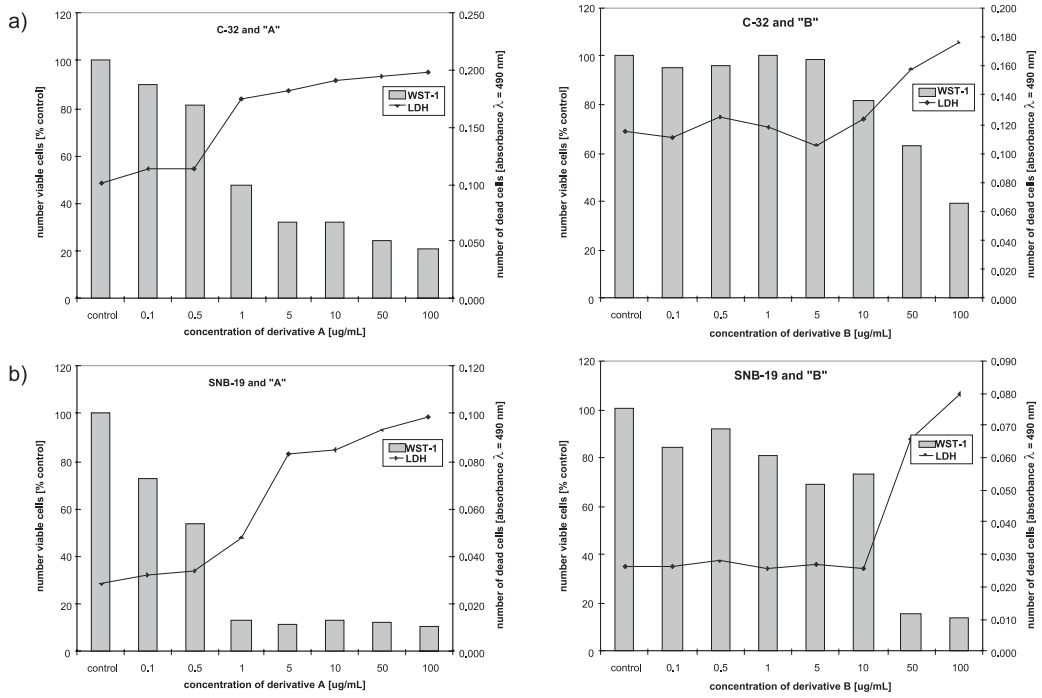

Figure 2. Cytotoxicity of A and B phenothiazines - number of cells in cultures of C-32 (a) and SNB-19 (b) after 72 h exposure to phenothiazine derivatives A and B. The number of viable cells (% of control) (WST-1 test) and the number of dead cells in the cultures (absorbance $\lambda = 490$ nm) (LDH assay). Statistical significance is indicated with star ($p \leq 0.05$).

tent. To establish cell number, WST-1 test (Roche Diagnostics GmbH, Germany) was used. Absorbance analysis were made by using UVM340 microplate reader (Biogenet, Poland).

Genes transcriptional activity: *H3, BCL-2, BAX, TP53, CDKN1A*

Genes trancriptional activity was evaluated by real time RT-QPCR method with OPTICON TM DNA Engine (MJ Research, USA) and QuantTect® SYBR® Green RT-PCR Kit (Qiagen GmbH, Germany). Genes analyzed were coding: histone H3 (*H3*), mitochondrial apoptosis pathway proteins (BCL-2 (*BCL-2*), BAX (*BAX*)) and cell cycle regulator protein P53 (*TP53*) and P21 (*CDKN1A*). Cells were exposed to examined phenothiazine derivatives in 0.5 µg/mL concentration for 24 h. RNA extraction was made using Quick-RNA™ Kit MiniPrep (ZYMO RESEARCH, USA). Quality and quantity of extracts was established. Total RNA integrity was analyzed in 1.2% agarose (PRONA S.A., Spain) electrophoresis with added ethidium bromide compound by using electrophoresis and blotting system - Mini-PROTEAN Tetra Cell and Mini Trans-Blot Module (BioRad, USA). Extracts total RNA quantity and purity were established using spectrophotometric analysis with HP845 spectrophotometer (Hewlett Packard, USA).

Antioxidative enzymes activity

Cells *in vitro* were exposed to examined phenothiazine derivatives in 0.5 µg/mL concentration for 24 h. As a next step, cells were harvested and lysed. After centrifugation, cell supernatant was stored on ice to analyze its total oxidative status (TOS) (18), superoxide dismutase activity (SOD) [EC 1.15.1.1] and malondialdehyde (MDA) concentration, which is lipids peroxidation indicator. SOD activity was measured by Oyanagui method (19), using its different susceptibility to cyanide inhibition, expressed as nitro units per gram of protein content. MDA concentration was measured by Ohkawa at al. method (20), using thiobarbituric acid reaction and results were shown as µmol of MDA per gram of protein content (21).

Statistical analysis

Statistical analysis was made using Statistica PL 9.0. Cell number in cell cultures and examined genes mRNA copy number after PCR reaction were

shown as an average of three replicates of three consecutive experiments with standard deviation (SD) content. Analysis used *t*-Student test for variables not dependent samples, Fisher test was used to count variance p factor, distribution normality was analyzed by Shapiro-Wilk test. Statistically significant changes were established on $p < 0.05$ relevance level.

RESULTS

Cell number analysis result in cell cultures exposed by 72 h to examined phenothiazine derivatives are shown in Figures 2 and 3. The examined phenothiazine derivatives cytotoxicity effect is visualised in Figure 2a (living and dead C-32 cell number in cell cultures exposed to A and B phenothiazine derivatives influence for 72 h) and Figure 2b (living and dead SNB-19 cell number in cell cultures exposed to A and B phenothiazine derivatives influence for 72 h). In both C-32 and SNB-19 cells, phenothiazine derivative A indicates more significant cytotoxic effect what WST-1 and LDH tests results confirm. SNB-19 have shown metabolically active cells level significant decrease starting with A derivative in 0.1 µg/mL concentration (WST-1). Also starting with 1 µg/mL derivative A concentration affecting SNB-19 cells, dead cell number increase (LDH) was observed. As described above, also melanoma C-32 cells indicate decreased viability and dead cell number increase while incubated with phenothiazine derivative A starting with 1 µg/mL concentration. In parallel, examined derivative B indicated cytotoxic effect starting with 50 µg/mL concentration (SNB-19) and 10 µg/mL concentration (C-32). Examined compounds affect cells proliferative ability by its significant decrease – derivative A already at a concentration of 0.1 µg/mL (C32 and SNB-19), derivative B at a concentration of 50 ug/mL (C32 and SNB-19) (Fig. 3). WST-1 analysis results seem to confirm those obtained by PCR analysis for phenothiazine derivatives in concentration 0.5 µg/mL, where *H3* histone (proliferation indicator) gene trancriptional activity decreases. Noticeable *H3* gene expression increase in SNB-19 examined with derivative B can derive from applied concentration in analyzed samples (Table 1). Copy number mRNA of analyzed *TP53* gene did not shown any significant changes between C-32 samples. In SNB-19 cells, derivative B presence caused significant *TP53* expression increase (Table 1). Phenothiazine B derivative also affected lowering P21 (*CDKN1A*) gene mRNA copy number in both C-32 and SNB-19 cells (Table 1). Despite visible cytotoxic effects in cultures exposed to A and B derivatives, any characteristic apoptotic genes *BAX* and *BCL-2* activity changes have not been observed (Table 1). Changes observed in increased ratio *BAX*/*BCL-2* mRNA copy number in examined samples, suggest cells protective antiapoptotic mechanisms activation (Table 1).

Preliminary cells TOS analysis results with phenothiazine A and B derivatives with 0.5 µg/mL supplemented media and MDA analysis results in both (melanoma nad glioma cells) indicate ongoing oxidative processes in examined samples (Table 2). Particularly evident are A derivative actions in C-32 cultures. SOD increased activity can be a confirmation of cell oxidative status changes for tested A and B compounds. The mentioned changes in SOD activity as one of major antioxidative enzymatic components, can also be a proof for protective

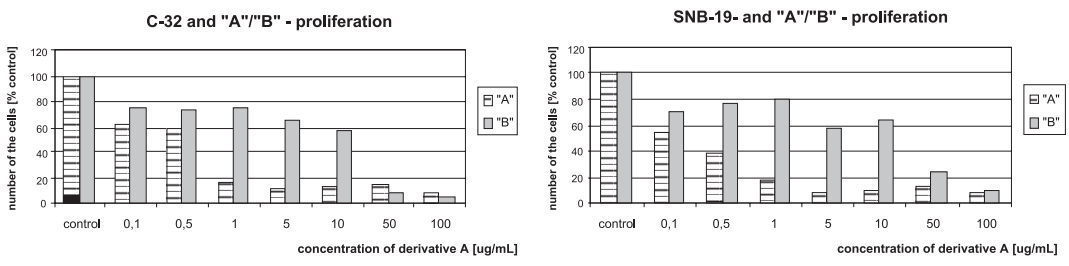

Figure 3. The proliferative activity of cells (C32 and SNB-19) - number of cells after 72 h exposure to the A and B derivatives of phenothiazine. Statistical significance is indicated with star ($p \leq 0.05$).

Table 1. A and B phenothiazine derivatives influence on *H3, TP53, CDKN1A* and *BCL-2, BAX* genes expression.

Gene	C-32			SNB-19		
	Number of mRNA copies/µg total RNA					
	Control	"A"	"B"	control	"A"	"B"
H3	183 ± 29	72 ± 15	123 ± 27	2436 ± 248	605 ± 53	56210 ± 4388
TP53	2892 ± 512	3130 ± 1302	2392 ± 283	1348 ± 293	3510 ± 751	144951 ± 21364
CDKN1A	3968 ± 732	5108 ± 964	660 ± 78	3294 ± 290	6500 ± 781	1605 ± 30
BCL-2	38675 ± 1652	68243 ± 23180	66416 ± 4258	2918 ± 396	4109 ± 799	17275 ± 3493
BAX	7350 ± 1652	2086 ± 848	3053 ± 571	26195 ± 4278	34356 ± 1355	53409 ± 7278
BCL-2/BAX	**5.26**	**32.71**	**21.75**	**0.111**	**0.120**	**0.323**

Table 2. Effect of phenothiazine A and B on the redox balance - TOS, MDA, SOD.

Gene	C-32			SNB-19		
	µmol/mg total protein					
	Control	"A"	"B"	control	"A"	"B"
TOS	0.82 ± 0.32	2.35 ± 1.11	1.96 ± 0.92	2.87 ± 0.85	3.53 ± 1.33	3.01 ± 1.53
MDA	24.33 ± 5.52	28.12 ± 8.52	25.34 ± 4.22	7.79 ± 2.31	14.62 ± 4.21	12.50 ± 7.32
	NU/mg total protein					
SOD	0.419 ± 0.122	1.280 ± 0.345	0.935 ± 0.423	0.383 ± 0.142	0.585 ± 0.131	0.461 ± 0.234

actions of SNB-19 and C-32 cells under examined conditions.

DISCUSSION and CONCLUSION

The aim of the study was to evaluate the influence of two amine derivatives of phenothiazine: A) 9-(*N*-piperidyl)-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride and B) 9-amino-5-methyl-12(*H*)-quino[3,4-*b*][1,4]benzothiazinium chloride on C-32 (ATCC) and SNB-19 (DSMZ) tumor cells *in vitro*. The analyzed molecules differ from each other by additional presence of quinobenzothiazinium chloride in amine group and piperidyl group at the 9- position of the quinobenzothiazinium ring.

Characteristic tumor cells feature is immortality and great proliferative activity which derives from impaired cell cycle regulation and control. As so far, existing knowledge says that phenothiazine derivatives can act as both cytotoxic and cytostatic compounds (2, 6, 7, 22-24). The examined cytotoxicity analysis of A and B derivatives have shown that in C-32 and SNB-19 cells more effectively acts A derivative (Fig. 2). WST-1 analysis was made to evaluate mitochondrial enzymes activity by specifying living cells number in cultures with media without FBS content. Such conditions prevented cell proliferation and where necessary for LDH analysis, where LDH enzyme is released by dead cells. First vital functions limitations effects were shown using 0.1 µg/mL derivatives concentration and 1 µg/mL concentration indicated statistically important dead cells number (Fig. 2). Fot both, A and B phenothiazine derivatives antiproliferative effect was also demonstrated. However, A substance indicated actions at lower concentration than B derivative (Fig. 3) in C-32 and SNB-19 cells. One of the anticancer drugs mechanisms of action is interaction with DNA what impedes proper cell metabolism and proliferation. Drugs affecting genes expression result in fully blocked replication. The mentioned mechanism was indicated by anthracycline antibiotics (doxorubicin) because of flat aromatic and heteroaromatic structural fragments. Also, so far existing knowledge suggests such antiproliferative effect in case of phenothiazines examinations (25, 26). In the present study, evaluated *in vitro* two chemical compounds A and B are phenothiazines analogues. Quinobezothiazinum salts X-ray analysis showed flat tetracycline structural compound (27), which attenuates intercalation process. Amine substituent present in the mentioned molecule may cause drug-DNA complex stabilization, what is possible due to

additional interactions of amino group *via* hydrogen bonds with purine and pyrimidine bases in the DNA molecule.

Antiproliferative activity of tested compounds for the C-32 cells has been confirmed in the analysis of expression of a gene encoding the histone H3 (Table 1). It is a generally accepted marker of proliferation in molecular studies. For SNB-19 cells, derivative B has increased the number of transcripts H3 (Table 1). Note, however, that the concentration of 0.5 ug/mL was used in these experiments (cytotoxic and antiproliferative effects appeared only at high concentrations - Figs. 2 and 3). The choice of concentration originated from the idea of comparing another experimental results not discussed it this study and also, A derivative concentration was limited by strong cytotoxic effect resulting in low living cells number at higher concentrations.

In the study, also BCL-2 (*BCL-2*), BAX (*BAX*), P53 (*TP53*) and P21 (*CDKN1A*) genes transcriptional activity was analyzed (Table 1). Real time RT-QPCR was made with QuantTect® SYBR® Green RT-PCR Kit (Quiagen) usage. Cells, as by histone *H3* analysis, were exposed to A and B derivatives in 0.5 µg/mL concentration.

TP53 gene product is P53 protein which acts as genome guardian and is also involved in many cell processes regulation. One of this protein major tasks is stopping cell cycle while DNA damages occur and when they are not repaired, P53 potein induce apoptosis. In cells, apoptosis can be initiated by intrinsic mitochondrial pathway with BCL-2 involved (pro- and antiapoptotic proteins family) or by extrinsic mitochondrial one were DR4 and DR5 receptors play an important role. P53 intracellular amount is strictly regulated by MDM2 protein (28). New target in anticancer therapies is to restore P53 activity in tumor cells what could lead to cancer cells precise degradation. This protein product regulates also protein P21 gene (*CDKN1A*) expression. This protein selectively binds to cyclin-dependent kinase complexes with cyclins and regulates cell cycle what means that inadequate P21 protein number or its mutations in cells can induce oncogenic transformation (29, 30). *TP53* and *CDKN1A* genes expression analysis performed in this study indicated significant increase of *TP53* expression only in SNB-19 glioma cells and phenothiazine derivative B examination (Table 1). It has been also shown that B derivative decreases mRNA copy number of P21 protein (*CDKN1A*). Obtained results and data give us new sight at actions of examined phenothiazine derivatives observed in cell cultures. However, it is important to draw attention that study applies only to examined genes mRNA transcripts. Facing changes in the expression of more genes, obtained results may not be precise. Some facilitation in the results interpretation are *BAX* and *BCL-2* genes expression analysis where *BAX*/*BCL-2* ratio is most important. Gene expression changes (among others P53 dependant) in proapoptotic *BAX* and antiapoptotic *BCL-2* ones do not indicate mitochondrial pathway cell number decrease dependence in cell cultures exposed to A and B derivatives. BAX protein role in cells lies in mitochondrial membrane permeability increase by pores formation therein, while BCL-2 protein is responsible for cytochrome C release into the cytosol (31, 32). As a result of described study, increased *BCL-2*/*BAX* ratio indicates protective mechanisms induction by examined cells before apoptosis pathway activation (Table 1).

The examined redox balance also has great importance for any cell proper functioning in living cells. Any disorders in this homeostasis can lead to oncogenic transformation but can be also used as a tool for its destruction and elimination from the organism (33-35). As obtained results have shown, C-32 exposition to A phenothiazine derivative (0.5 µg/mL) cause significant TOS changes and promotes oxidative activity (increased MDA concentration) in examined cells. However, SNB-19 indicate oxidative promotion by examined substances on lower level than in C-32 cells (Table 2). Also, SOD increased activity can confirm oxidative cell status in phenothiazine derivatives presence because of its great involvement in antioxidative protective actions as one of major enzymatic systems.

In conclusion, A and B phenotiazine derivatives influence tumor cells viability and chosen molecular markers in C-32 and SNB-19 cells. Analyzed molecules differ from each other by additional presence of quinobeznothiazinium chloride in amine group and piperidyl group at the 9-position of the quinobenzothiazinium ring. Both analogues cause multiple metabolic changes in C-32 and SNB-19 cells, what results in viability and proliferation limitations. Although A derivative indicated stronger effect of action, both A and B analogues seem not to affect BCL-2/BAX signalling pathway irregularities. Study results have shown that amine heterocyclic compound in quinobenzothiazinium chloride molecule increases antiproliferative activity in both examined cell lines. This result is very interesting and encourages studies focusing on the new qiuonobenzothiazinium salts synthesis as new potential anticancer drugs.

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THE EFFECT OF SULFASALAZINE AND 5-AMINOSALICYLIC ACID ON THE SECRETION OF INTERLEUKIN 8 BY HUMAN COLON MYOFIBROBLASTS*

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Abstract: Sulfasalazine (SAS) and its therapeutically active derivative - 5-aminosalicylic acid (5-ASA) are used in the treatment of inflammatory bowel disease. 5-ASA mechanism of action on the one hand, involves the inhibition of the cyclooxygenase and lipoxygenase activity, and thus decrease of synthesis of prostaglandins, leukotrienes and free radicals, on the other hand, the suppression of the immune response in the intestinal mucosa. Myofibroblasts, which are located just below the basement membrane, are important element of the mucosa. Due to its secretory activity they may interact with other cells, including epithelial cells. Examining SAS and 5-ASA cytotoxic properties on human normal, colon subepithelial myofibroblasts (CSEMF) it was found that the first of these compounds in a concentration of 1 mM significantly reduced the number of these cells as compared to the control, while the latter exhibited an action at the 5-fold higher concentration (5 mM). Moreover, SAS concentration greater than 0.25 mM reduced IL-8 secretion by CSEMF, and 5-ASA had no effect in the tested range of concentrations, i.e., up to 7.5 mM.

Keywords: sulfasalazine, 5-aminosalicylic acid, interleukin 8, colon subepithelial myofibroblasts

Currently, gastrointestinal diseases are lifestyle diseases. The diet of modern man influences the development of inflammatory bowel disease (IBD), which includes, among others ulcerative colitis (UC) and Crohn's disease (CD). Drug of first choice in these conditions is sulfasalazine (SAS) and its active metabolite 5-aminosalicylic acid (5-ASA). Despite intensive research conducted by many reputable research centers, the mechanism of action of these drugs is still not fully recognized. Sulfasalazine exhibits bacteriostatic, anti-inflammatory and immunosuppressive activity. The therapeutic effect of SAS results from the action of its pharmacologically active derivative 5-ASA, while the other derivative - sulfapyridine - is a carrier preventing the absorption of salicylic derivative in the small intestine (1). 5-ASA influences the metabolism of arachidonic acid (2-4). The mechanism of this process is not fully clear, since neither cyclooxygenase inhibitors affect the course of inflammation in IBD, nor the effectiveness of lipoxygenase inhibitors is comparable to that of 5-ASA. Therapeutic effects probably result not only from the reduction of the synthesis of inflammatory mediators such as leukotrienes (LT4), prostaglandins and PAF, but also from inhibition of the migration of inflammatory cells into the intestinal mucosa and immunoglobulin production by B cells. The inhibition of cytokine (IL-1, TNF-α, INF-α) secretion and the abolition of chemotactic action of formylated bacterial peptides, which are probably responsible for the migration of polymorphic mononuclear cells to intestinal mucosa, is also observed (5).

A chronic, relapsing bowel inflammation is accompanied by increased production of pro-inflammatory cytokines, increased permeability of the intestinal epithelium and changes in the synthesis of mucus. Epithelial cell functions such as proliferation, differentiation, cytokine secretion, motility and permeability can be regulated, among others, by myofibroblasts (6). These cells, participating in the regulation of local inflammatory processes, synthesizes many cytokines. Not only do they secrete pro-

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inflammatory cytokines such as IL-1, IL-6, IL-8, RANTES (regulated on activation, normal T-cell expressed and secreted), monocyte chemotactic protein-1 (MCP-1), but also anti-inflammatory cytokines such as IL-10 (7, 8).

The aims of study was to investigate the influence of SAS and 5-ASA on colon subepithelial myofibroblasts (CSEMF) viability and the secretion of IL-8 by these cells stimulated by TNF-α.

EXPERIMENTAL

Cell cultures

Normal human colon myofibroblasts CCD-18Co were obtained from American Type Culture Collection. The cells were cultured in minimum essential medium (MEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Sigma), 100 IU/mL penicillin G, 100 mg/mL streptomycin and 10 mM HEPES (Gibco). The cell cultures were maintained at 37°C in 5% CO_2 atmosphere.

Cytotoxicity assay

The XTT (*In Vitro* Toxicology Assay Kit XTT Based, TOX-2, Sigma) assay was used to assess cell viability. The method is based on the ability of mitochondrial dehydrogenases of viable cells to cleave the tetrazolium ring of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt), yielding orange formazan crystals, which are soluble in aqueous solutions. Myofibroblasts were dispensed at a density of 1000 cells / 0.2 mL into 96-well plates and were cultured in MEM supplemented with 10% FBS for 72 h. After this time, the cells were washed three times with RPMI (without phenol red dye), then 150 μL of XTT with 0.1, 0.25, 0.5, 1 mM SAS and 0.5, 1, 2.5, 5 mM 5-ASA solution was added into each well for 4 h. The absorbance of samples was measured at 450 nm (reference 690 nm) using a plate reader (MRX Revelation, Dynex). The absorbance was directly proportional to the amount of the living cells.

IL-8 assay

Myofibroblasts were dispensed at a density of 5000 cells / 0.2 mL into 96-well plates. The cells were grown 4 days in MEM supplemented with 10% FBS. Twenty four hours before initiation of the proper experiment, the medium was replaced with a medium containing 1% FBS and then the cells were cultured with 0.1, 0.25, 0.5, 1 mM SAS and 1, 2.5, 5, 7.5 mM 5-ASA for 24 h in present of 37.5 ng/mL TNF-α. After this time, the supernatants of the cells were frozen in -70°C and the amount of living cells was evaluated with the XTT test.

The concentrations of IL-8 in the supernatants (diluted 1 : 10) were determined by ELISA MAX™ according to the instructions of the manufacturer (Biolegent). The absorbance at 450 nm (570 nm references) was measured with the plate reader (MRX Revelation, Dynex). Quantity IL-8 [ng] was determined from a standard curve and the resulting values were calculated per 10^6 of living cells.

Statistics

To evaluate the influence of SAS and 5-ASA on the secretion of IL-8 by myofibroblasts, the arithmetic mean as a measure of the average and standard deviation as a measure of dispersion were used.

Differences in IL-8 secretion were analyzed for statistical significance using analysis of variance (ANOVA) and Tukey test. The results of SAS and 5-ASA cytotoxicity test were analyzed by Kruskal-Wallis and U-Mann-Whitney test. Normality was verified by Shapiro-Wilk test, and homogeneity of variance by Brown-Forsythe test. A p-value < 0.05 was considered significant. The analysis was performed using Statistica 10.0 software (StatSoft, Poland).

RESULTS AND DISCUSSION

The SAS mechanism of action is still under discussion. Putative anti-inflammatory action of 5-ASA includes the modulation of cytokines secretion, the inhibition of macrophage activation, induction of apoptosis, reduction of the transcriptional activity of the nuclear transcription factor (NF-κB), inhibition of prostaglandins and leukotrienes biosynthesis, the interaction with the peroxisome proliferator-γ activated receptor (PPAR-γ), inhibition of neutrophil chemotaxis and the influence on reactive oxygen species (ROS) (9, 10).

Sulfasalazine also inhibits the synthesis of a number of pro-inflammatory cytokines, i.e., interleukin-1, -2, -12 (IL-1, IL-2, IL-12), tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), reduces the number of B cells and decreases the antibodies synthesis. It reduces the metabolism of granulocytes (11). SAS influences NK (natural killer) cells, epithelium and endothelium cells, neutrophils, T cells and macrophages (10). Colon subepithelial myofibroblasts (CSEMF) regulate local inflammatory processes. They secrete both pro- and anti-inflammatory cytokines, chemokines, growth factors, mediators of the inflammatory response and are the source of reactive oxygen species (12, 13). Di

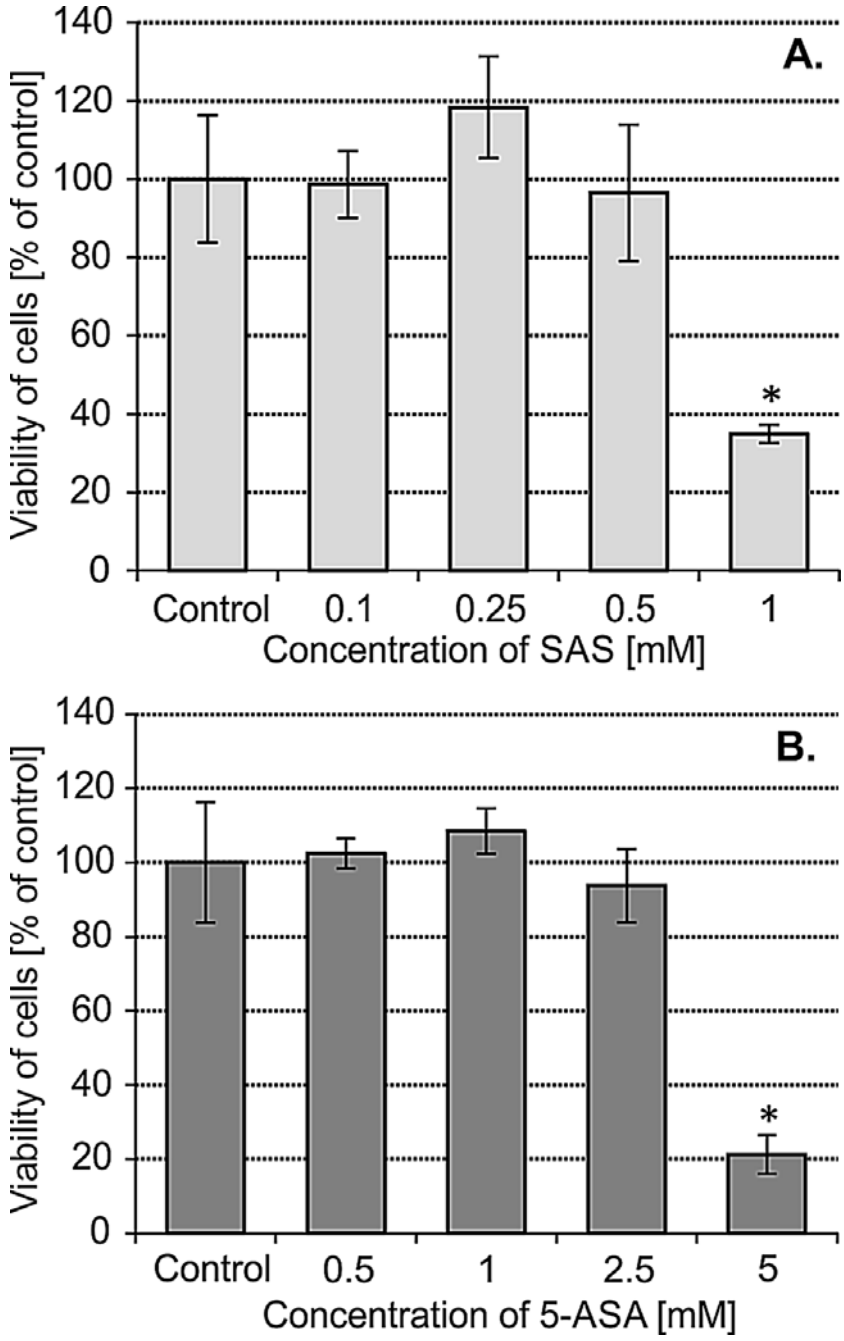

Figure 1. Cytotoxicity of SAS (A) and 5-ASA (B) towards human normal colon myofibroblasts after 72 h of incubation (*$p < 0.05$ compared with untreated control)

Sabatino et al. (14) found in patients suffering IBD the increased levels of TNF-α, which interfering with the target cell receptor leads to activation of the NF-κB pathway. Since TNF-α and IL-1 can induce NF-κB forming a positive autocrine loop, it may be supposed that NF-κB is crucial factor in the pathogenesis of chronic inflammation (15, 16). SAS inhibit PMA-, TNF-α-, or LPS-induced activation of NF-κB (11), that induces apoptosis, probably by preventing the expression of anti-apoptotic genes.

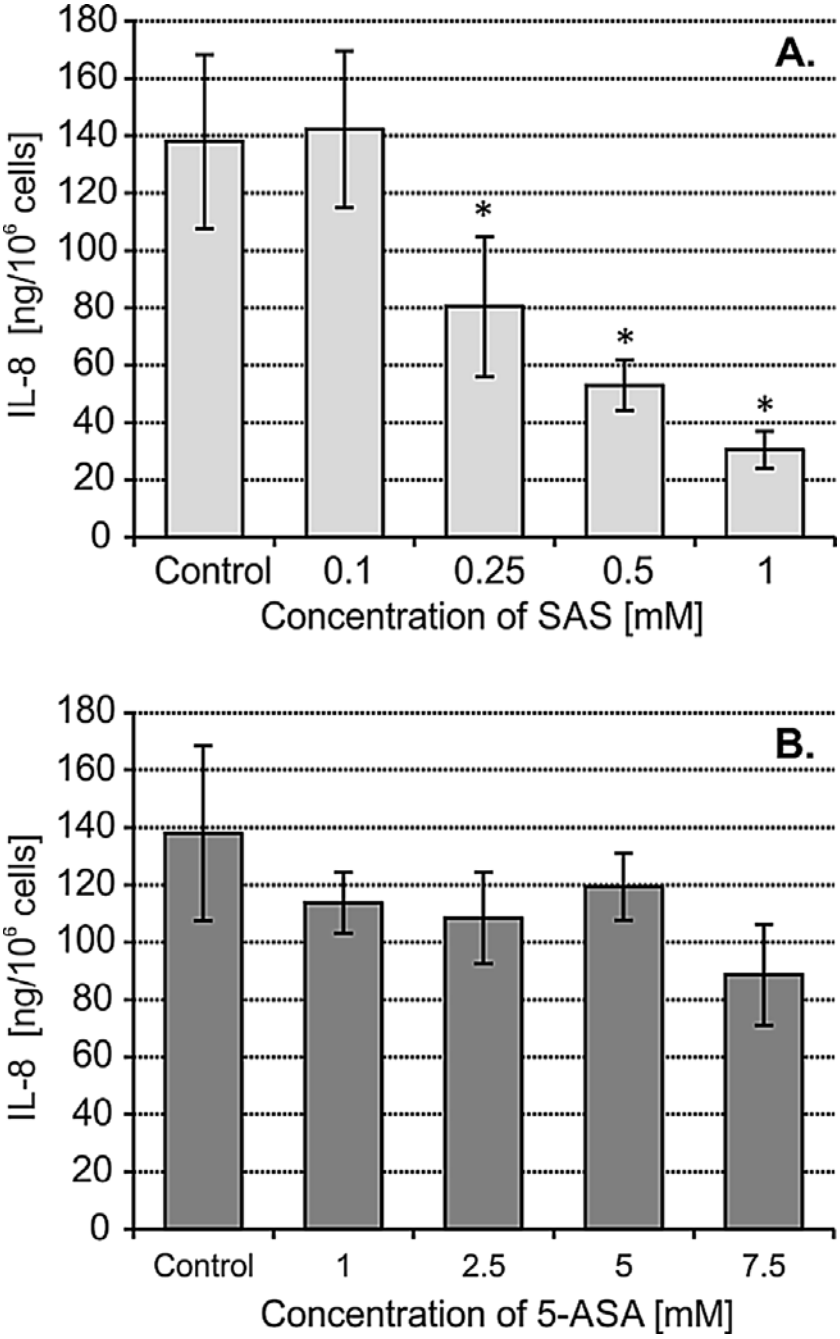

Figure 2. Influence of SAS (A) and 5-ASA (B) on IL-8 secretion by TNF-α-stimulated human colon myofibroblasts (*$p < 0.05$ compared with untreated control)

IBD is associated with extremely low level of cells apoptosis at the site of inflammation. In a healthy organism anergy and apoptosis in T cells occurs. In intestinal mucosa apoptosis is intense, while during the CD this process is reduced, which is associated with excessive production of anti-apoptotic cytokines IL-2, IL-6, IL-15, IL-17 or IL-18 (17).

In this paper, colon myofibroblasts survival in the presence of SAS and 5-ASA was shown as a percentage of control. The study showed that SAS at a concentration of 1 mM significantly reduces the CSEMF number as compared to the control ($p < 0.0353$, Kruskal-Wallis test) (Fig. 1A), while 5-ASA exhibits cytotoxicity at 5-fold higher concentration

(5 mM) ($p < 0.0158$, Kruskal-Wallis test) (Fig. 1B). Myofibroblast proliferation is enhanced at the edges of ulcers of IBD patients, helping in wound healing. Thus, observed cytotoxicity of the investigated compounds has probably negative impact on this process.

The study assessed the effect of the SAS and 5-ASA on the secretion of IL-8 by TNF-α-stimulated human CSEMF. Statistically significant reduction of this cytokine secretion was observed at a concentration equal to or higher than 0.25 mM of SAS, while 5-ASA did not show such an effect at tested concentrations (up to 7.5 mM). 5-ASA affects intestinal inflammation by inhibition the secretion of IL-8 by macrophages and monocytes (18). There is no constitutive secretion of IL-8 by human CSEMF, however, when IFN-γ treated, they start to secrete this cytokine. IL-8 is also secreted by LPS stimulated CSEMF (19). Our findings showed that similar effect is observed when these cells are stimulated by TNF-α. IBD patients IL-8 increased secretion by the ISEMF is due to the increased IL-17 secretion by T cells, predominantly IL-23-dependent Th17 subpopulation. This interleukin is composed of two protein subunits p40 and p19, which is found in myofibroblasts (20). In the mucosa of IBD patients increased secretion of IL-22 is also observed, that results in the enhanced expression of IL-8 by the target cells.

CONCLUSION

SAS up to concentration of 0.5 mM had no influence on CSEMF viability, simultaneously decreasing TNF-α-dependent IL-8 secretion. There is no effect of 5-ASA on IL-8 secretion by CSEMF at investigated concentrations of this compound. Therefore, low doses of SAS may support the IBD treatment with 5-ASA.

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ANTIPROLIFERATIVE AND CYTOTOXIC EFFECT OF SELECTED VITAMIN D ANALOGS ON NASAL POLYPS FIBROBLASTS AND OTHER CELLS WITH HIGHER PROLIFERATIVE POTENTIAL*

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Abstract: Besides well-known effect on bone and mineral metabolism vitamin D is involved in essential non-calcemic regulatory mechanisms, such as cellular proliferation, differentiation and apoptosis in various cell types. Major limitation for therapeutic use of calcitriol, a hormonally active form of vitamin D, is its calcemic and phosphatemic action. Recently, more selective vitamin D analogs which retain clinically useful activities with reduced toxicity have been designed. The aim of the present study was to evaluate the *in vitro* effect of vitamin D analogs on proliferation rate and survivability of cells with increased proliferative activity. The effect of calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 was examined. The experiments were performed on cultures derived from nasal polyps and cancer cells lines (SNB-19, C32 and SH-4). Cultures were incubated 72 h with tested compounds, each at the concentration of 0.025, 0.25, 2.5 and 25 μg/mL. The cytotoxic effect of vitamin D analogs and their influence on growth rate were determined using WST-1 assay. RT-QPCR technique was used to evaluate the expression of anti-apoptotic *BCL-2* and pro-apoptotic *BAX* gene.
Each of the tested compounds presented significant effect at the concentrations above 0.25 μg/mL. The strongest inhibition of the growth rate and decrease in cell survivability was observed after treatment with PRI-1890 and PRI-2191. Stimulation with calcitriol and other vitamin D analogs led to decrease *BCL-2*/*BAX* mRNA ratio in each cell lines. The apparent pro-apoptotic action revealed PRI-2191 followed by PRI-1890. It might be hypothesized that vitamin D analogs supplementation may provide therapeutic benefits not only in oncological patients but also in chronic rhinosinusitis.

Keywords: vitamin D analogs, cytotoxicity, growth rate, apoptosis, nasal polyps, cancer, *BAX*, *BCL-2*

Besides well-known effect on bone and mineral metabolism vitamin D (VD) is involved in essential noncalcemic regulatory mechanisms, such as cellular proliferation, differentiation and apoptosis in various cell types (1). VD plays also an integral role in angiogenesis and number of immunologic effects towards lymphocytes, dendritic cells and macrophages (2). Calcitriol (1,25-dihydroxyvitamin D3 or 1,25-dihydroxycholecalciferol) is a hormonally active form of VD made from calcidiol in kidneys and other tissues or delivered with food (Fig. 1). The most important downstream signal transduction pathways activated by calcitriol in target cells are realized though so called "genomic pathway" involving specific nuclear VD receptor (VDR). Activation of VDR leads to the relaxation of chromatin structure making DNA accessible for RNA polymerase and subsequent initiation of transcription of target genes (3).

In vitro and *in vivo* observations have demonstrated that calcitriol is a potent inhibitor of cells growth (4). Anti-proliferative and pro-differentiating properties of 1,25-dihydroxyvitamin D3 would make it a promising element of anticancer therapies (5). Major limitation for therapeutic use of calcitriol is its calcemic and phosphatemic action. The adverse effects motivated the search for new semi-selective analogs (VDA) which retain clinically useful activities with subsequent reduced calcium mobilizing potential.

Due to the multidirectional activities and lower toxicity, supplementation of newer VDA is hypothesized to have a positive therapeutic effect also in chronic inflammatory diseases (6). Previously, we

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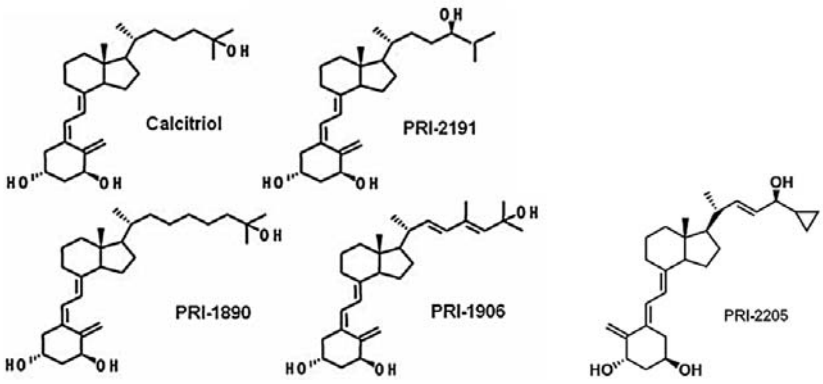

Figure 1. Structures of calcitriol and its analogs: PRI-2191, PRI-1890, PRI-1905 and PRI-2205.

have presented potential applicability of tacalcitol (PRI-2191) in the prevention and treatment of chronic rhinosinusitis with nasal polyps (NP) (7).

Chronic rhinosinusitis (CRS) is a common disease with a substantial health care impact. Due to the complex and unclear etiology, CRS remains unresolved and presents difficult therapeutical challenge. Due to well-known side effects related to steroids, there is a need for the investigation of new agents suitable for CRS management. Mulligan et al. (8) showed that patients with CRS demonstrate insufficient level of VD when compared to the control. Supplementary treatment of CRS involving VDA would be similar to broad spectrum of dermatological conditions where a moderate to strong recommendation was given for the use of topical VD in combination with steroids (9). Additionally, there is constant need to study the effectiveness of particular, newly designed analog especially in terms of unknown structure-function relationship.

The objective of this study was to determine the effect of VDA on growth rate and survivability of selected cells with increased proliferative activity (i.e., nasal polyps' fibroblasts, SNB-19, C32 and SH-4 cell line). VDA included calcitriol, tacalcitol [(24*R*)-1,24-dihydroxyvitamin D3 or PRI-2191] and three newer compounds marked as PRI-1890, PRI-1906 and PRI-2205 (Fig. 1).

EXPERIMENTAL

Cells culture and treatment

The experiments were performed on the following cells: fibroblasts derived from NP, astrocytoma cell line SNB-19 (DSMZ no. ACC 325), amelanotic melanoma cell line C32 (ATCC® CRL-1585™) and melanoma cell line SH-4 (ATCC® CRL-7724™). NP samples were obtained from 3 patients with CRS during routine surgical procedure performed in the Dept. of Otolaryngology, Wroclaw Medical University in Poland. All the subjects met the diagnostic criterion for CRS as established by the European position paper on rhinosinusitis and nasal polyps (EPOS 2012). Patients had been free of any medications for at least 4 weeks before surgery and had bilateral NPs on endoscopic examination and computed tomography (CT). The presence of the comorbidities was excluded. The study was approved by Local Ethical Committee of Wroclaw Medical University.

NP specimens were immediately rinsed in phosphate buffered saline (PBS), cut into small fragments and placed into a sample tubes containing 1 mL PBS. The tubes were directly transported on ice to the laboratory for further investigations. A part of each sample was fixed in 10% buffered neutral formalin (Chempur, Piekary Śl., Poland), processed routinely, and embedded in paraffin wax (Bio-Plast, Wrocław, Poland) for subsequent immunohistochemical examination to establish diagnosis and to exclude other pathologies.

Survivability assay

Cultures were set and carried out in Nunc 75 mL non-treated cell culture flasks. Cells were seeded at 10,000/well in a 96-well plate and incubated for next 24 h. Cells grown in DMEM (Lonza, Basel, Switzerland) medium supplemented with 10% fetal bovine serum (FBS) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel), penicillin (10000

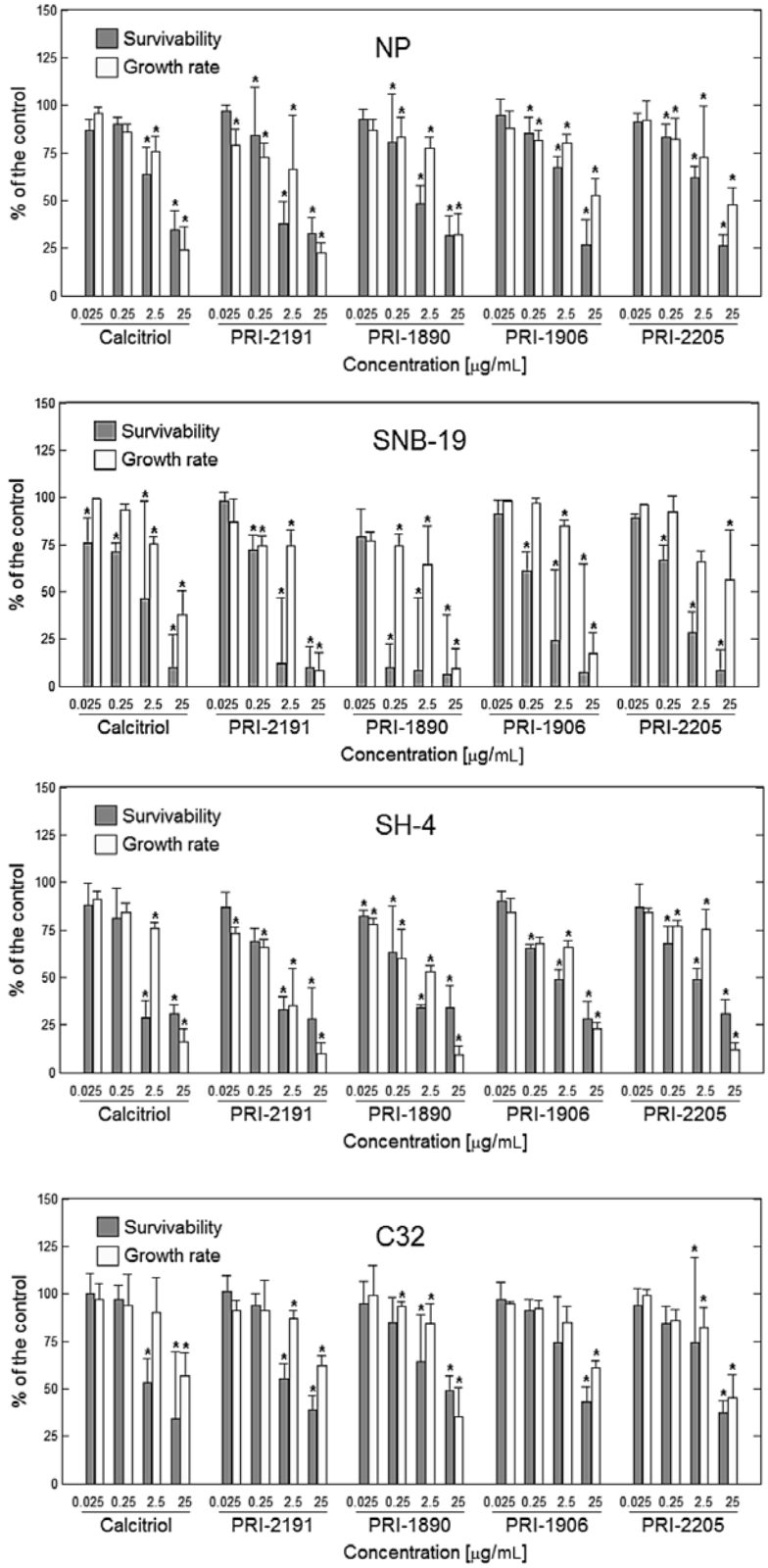

Figure 2. The results of WST-1 assay to determine cells survivability and proliferation rates of NP fibroblasts, SNB-19, SH-4 and C32 cells treated with calcitriol and vitamin D analogs (PRI-2191, PRI-1906, PRI-2205 and PRI-1890). Data are shown as the mean percentages (± SD) of the control culture. *Significant difference from the respective control culture ($p < 0.05$)

U/mL) and streptomycin (10 mg/mL) (Biological Industries Ltd., Kibbutz Beit-Haemek, Israel).

Restriction of cells viability (resulting from the cytotoxicity of VDA) was assessed in cultures carried out in medium without growth stimulating factors necessary for cell divisions. For that reason, the WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany) was performed after 72 h cells incubation with calcitriol and VDA (PRI-2191, PRI-1890, PRI-1906 or PRI-2205) (Fig. 1) at 0.025, 0.25, 2.5 or 25 μg/mL in FBS-free medium, according to the manufacturer's protocol. Calcitriol and VDA were manufactured and certified by the Pharmaceutical Research Institute (Warszawa, Poland). Control wells were filled with a medium without calcitriol and VDA. WST-1 assay is based on reduction of a substrate (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) to formazan. Reduction in enzyme activity leads to a decrease in the amount of formazan dye, which directly correlates to the number of metabolically active cells in the culture. The intensity of the colorimetric reaction was measured by microplate reader UVM340 (Biogenet, Piaseczno, Poland) at 440 nm.

Growth rate assay

To asses the influence of calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 on growth rate tested cells were incubated for 72 h in medium containing FBS and further supplemented with each agent at various concentrations (0.025, 0.25, 2.5 and 25 μg/mL). Total number of living cells in the cultures was estimated by cytotoxicity-proliferation WST-1 assay (Roche Molecular Biochemicals, Mannheim, Germany). Control wells were filled with a medium without calcitriol and VDA under the same conditions.

Determination of transcriptional activity of *BAX* and *BCL-2* genes

In order to evaluate the expression of anti-apoptotic *BCL-2* gene and pro-apoptotic *BAX* gene QRT-PCR technique was used. The cells were first exposed for 24 h to calcitriol, PRI-2191, PRI-1890, PRI-1906 and PRI-2205 at 0.25 μg/mL. Extraction of RNA from the cells was performed using Quick-RNA™ MiniPrep Kit (Zymo Reseach, Irvine, CA, USA). To determine the transcriptional activity of *BCL-2* and *BAX* gene the fluorescence detector DNA Engine OPTICON™ (MJ Research, San Francisco, USA) and set of reagents from QuantiTect™ SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) were used.

Statistical analysis

Statistical analysis was performed using Statistica PL 7.0 (Statsoft, Kraków, Poland). The data were presented as the mean ± SD. Each experimental sample was at least in triplicate and each experiment was performed at least three times. The differences between groups were explored with ANOVA and *post-hoc* Duncan test was applied. A level of $p < 0.05$ was considered to be significant.

RESULTS

Influence of calcitriol and other VDA on survivability of the cells

Calcitriol and VDA presented cytoxic effect towards cells in each of the tested colony carried out in FSB-free medium (Fig. 2). A lack of growing stimuli (FBS) limited cells divisions within the cultures. Calcitriol and VDA action was dose-dependent. Dose dependency was most clearly visible in SNB-19 and SH-4 cell lines. Calcitriol and VDA significantly decreased cells viability even in lower concentration i.e., starting at 0.025 μg/mL in SNB-19, SH-4 and NP cell lines. In C32 cell line calcitriol and VDA had to be applied in higher concentrations ≥ 0.25 μg/mL) to obtain the same results. Susceptibility to the cytotoxic action of calcitriol and VDA in the cell lines showed origin-specific manner. SNB-19 cells were the most susceptible, whereas C32 were the least sensitive to VD compounds. PRI-1890 and PRI-2191 restricted cells viability the most efficiently independently from cells origin.

Modulation of cells' growth by calcitriol and other VDA

In the second part of the experiment, the influence of calcitriol and VDA on the cells growing under the conditions enabling cell divisions were assessed. Such conditions made it possible to evaluate not only cytotoxic properties of VDA but also the impact of calcitriol and VDA on cell cycles progression and cells divisions. The action of calcitriol and VDA in each cell line was dose-dependent. Under those conditions, calcitriol and VDA efficacy to decrease cells number was weaker in most of the concentrations compared to that obtained in FSB-free medium. The exception included SH-4 cell line where calcitriol and other VDA at the highest concentration used (25 μg/mL) suppressed cells number more significantly than in FSB-free medium. The same results were obtained with PRI-1890 and PRI-2191 at 25 μg/mL in C32 and SNB cell lines, respectively. In NP fibroblasts, PRI-1890 (at 0.025,

0.25 and 25 μg/mL), PRI-2191 (at 25 μg/mL) and PRI-1906 (at 0.025 and 0.25 μg/mL) impaired growth rate more efficiently.

Modulation of *BCL-2* and *BAX* gene expression by calcitriol and VDA

Calcitriol and its analogs were used at 0.25 μg/mL each to asses the influence on the expression of pro-apoptotic *BAX* and anti-apoptotic *BCL-2* gene. The results were showed in a form of *BCL-2*/*BAX* ratio which better reflects the apoptosis process than isolated *BCL-2* and *BAX* expression levels (Fig. 3). Stimulation with calcitriol and other VDA led to a decrease of *BCL-2*/*BAX* mRNA ratio in each tested cell lines. The strongest pro-apoptotic action had PRI-2191 followed by PRI-1890.

DISCUSSION AND CONCLUSION

In the present paper we investigated the cytotoxic and anti-proliferative effects of calcitriol and VD analogs in selected cancer cell lines and fibroblast derived from nasal polyps. Although the tested compounds were differentially active in different cell lines, PRI-1890 and PRI-2191 restricted cells viability most efficiently, independently from cells origin. It might be suggested that PRI-1890, PRI-2191 and PRI-1906 at the higher concentrations may additionally impair cells number not only due to their cytotoxic activity but also through the modulation of the cell cycle progression and divisions.

According to the published data, the therapeutic efficacy of calcitriol and many VDA, as single agents, for the treatment of cancers has not yet fulfilled its promise. However, newer VDA including PRI-2191, PRI-1906, PRI-2205 and PRI-2202 were seen to be more potent inhibitors of cancer cells proliferation than calcitriol in monotherpy or in combination with cyclophosphamide or cisplatin (10, 11). PRI-2205, a geometric analog of calcipotriol, which exerted antiproliferative activity *in vitro* and antitumor activity *in vivo*, revealed less calcemic at the doses which inhibit tumor growth (12). Similarly, PRI-1906, VDA with the extended and rigid side chain has higher antiproliferating activities with lower risk of hypercalcemia and toxicity than calcitriol. In particular, Baurska et al. (13) observed approximately 30% inhibition of proliferation in prostate cancer cells in response to PRI-1906. From the experiments carried on AML derived cells it is known that PRI-1906 has stronger than 1,25-dihydroxyvitamin D3 pro-differentiating activities. Antiproliferative potency of VDA has not been clearly explained, although the Cdk inhibitor $p27^{Kip1}$ seems to be important in that process (14). It was showed also that calcitriol induced cell cycle arrest is accompanied by decreased Cdk inhibitor $p21^{Kip1}$ expression and Rb dephosphorylation what further inactivates members of E2F family of transcription factors (15, 16). The outcomes of those studies showed distinct difference between the action of the compounds and the origin of cells what agrees with our results. While in cancer therapy pro-differentiating properties of VD seems to be essential, in the case of treatment of inflammatory diseases antiproliferative, pro-apoptotic and immunomodulating

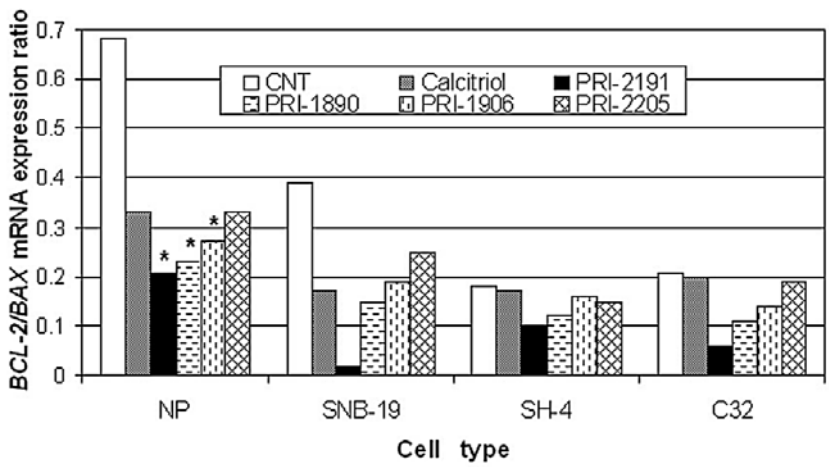

Figure 3. Effect of calcitriol and other vitamin D analogs (PRI-2191, PRI-1906, PRI-2205 and PRI-1890) on the *BAX*/*BCL-2* mRNA ratio in NP fibroblasts, SNB-19, SH-4 and C32 cells lines. Values are the means ± SD. *Significant difference from the respective control culture ($p < 0.05$)

activity would be much more important. Our previous study showed that tacalcitol appeared to be more active in inhibition of NP fibroblasts proliferation than calcitriol (7).

The way in which VDA modulate apoptosis is dependent on the type of cells to be treated and is not apparent. McGuire et al. (17) reported that the pro-apoptotic effect of 1,25-dihydroxyvitamin D3 in murine squamous cell cancer is associated with up-regulation of MEKK-1. Although downstream pathway of MEKK-1 that leads to apoptosis remains undefined, there is evidence that MEKK-1 activation results in conformational changes in the pro-apoptotic BAK protein (18). Other publication indicates the role for 1,25-dihydroxyvitamin D3 in alteration of BAX subcellular distribution in the pro-apoptotic effect (19). Previously, we have revealed slightly better efficiency of tacalcitol over calcitriol in reducing *BCL-2*/*BAX* mRNA ratio in fibroblasts derived from NP (20). Herein, calcitriol and other tested VDA decreased *BCL-2*/*BAX* mRNA ratio in each cell line compared to the respective control. Tacalcitol (PRI-2191) showed the strongest pro-apoptotic activity among the all tested compounds. However, PRI-1890 revealed very similar pro-apoptotic efficacy in NP fibroblasts, SH-4 and C32 cells. Stronger pro-apoptotic activity of PRI-2191 and PRI-1890 may in part explain the pronounced effect of both compounds limiting cells survival observed in our study. PRI-2205 has been shown before to cause apoptosis of HL-60 cells at a dose of 10 nM, but at a higher dose (100 nM) it caused cells differentiation (10). Contrary, according to Dehghani et al. (21), 1,25-dihydroxyvitamin D3 may suppress cells apoptosis as it was showed in multiple sclerosis patients.

Our and other studies performed on the anti-inflammatory effects of VDA suggest the possibility of their application in CRS to develop treatment strategies that include the use of steroids in combination with either VDA or phototherapeutics. Few of the same pathways affected by dexamethasone overlapped with the VD responses (22). Additionally, it was observed that VDA stimulate innate immunity and enhance antimicrobial activity by upregulating synthesis of the anti-infective molecules like cathelicidin (23). Xystrakis et al. (24) revealed that administration of VDA enhance subsequent responsiveness to dexamethasone for induction of IL-10 what indicates that VDA could potentially increase the therapeutic response to steroids in CRS.

On the basis of the present study it might be hypothesized that vitamin D analogs have a therapeutic function not only in selected neoplastic disorders but also in chronic inflammatory diseases such chronic rhinosinusitis.

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DRUG SYNTHESIS

SYNTHESIS AND ANTIINFLAMMATORY ACTIVITY OF SOME IMIDAZO[2,1-*b*][1,3,4]THIADIAZOLE DERIVATIVES

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Abstract: A number of imidazo[2,1-*b*][1,3,4]thiadiazole derivatives having alkyl and aryl moieties attached to positions 2 and 6 of imidazo[2,1-*b*][1,3,4]thiadiazole nucleus, respectively, were prepared and characterized by IR, NMR and mass spectroscopy. Antiinflammatory activity was evaluated by carrageenan-induced rat paw edema assay. By 5th hours, all compounds demonstrated anti-inflammatory activity similar or higher than that of standard NSAID, ibuprofen.

Keywords: imidazo[2,1-*b*][1,3,4]thiadiazole, antiinflammatory activity, carrageenan

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (1). Inflammation is a protective attempt by the organism to remove the injurious stimuli and to initiate the healing process. Inflammation is not a synonym for infection, even in cases where inflammation is caused by infection. Although infection is caused by a microorganism, inflammation is one of the responses of the organism to the pathogen. Without inflammation, wounds and infections would never heal. Similarly, progressive destruction of the tissue would compromise the survival of the organism. However, chronic inflammation can also lead to a host of diseases, such as hay fever, periodontitis, atherosclerosis, rheumatoid arthritis, and even cancer (e.g., gallbladder carcinoma). It is for that reason that inflammation is normally closely regulated by the body.

Non-steroidal antiinflammatory drugs (NSAIDs) are the most commonly prescribed medications in the world. They are used for the treatment of pain, fever and inflammation, particularly arthritis (2, 3). The most prevalent side effects of the use of non-steroidal antiinflammatory drugs are the occurrence of gastrointestinal damage with gastric upset and irritation being the major problems (4, 5). The search for safer NSAIDs has intensified and continues with the failure of anticipated 'ideal' anti-inflammatory agents, the coxibs, on long-term usage (6, 7).

During recent years, there have been an intense investigations on imidazo[2,1-*b*][1,3,4]thiadiazole compounds, many of them are known to possess interesting biological properties such as anticonvulsant (8, 9), antimicrobial (10, 11), anti-inflammatory (12, 13), antitubercular (14, 15), antihypertensive (16, 17) and anticancer (18) activities. In view of the above and in continuation of our research on fused imidazo[2,1-*b*][1,3,4]thiadiazoles, we report here the anti-inflammatory activity of some 2-(4'-substituted benzyl)-6-phenylimidazo[2,1-*b*][1,3,4]thiadiazoles.

EXPERIMENTAL

Chemicals and reagents

All the chemicals used in the present study were of analytical grade and purchased from Sisco

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Research Laboratory (SRL), India. The IR spectra were recorded in KBr on a Jasco 430+ (Jasco, Japan), the ^{1}H NMR spectra were recorded in DMSO-d_6 on a Bruker (400 MHz) (Bruker, Germany) aparatus, and *J* values are reported in Hertz. The melting points are uncorrected. Silica gel plates were used for the TLC by using $CHCl_3$: MeOH (9:1, v/v) mobile phase.

General procedure for the preparation of 2-amino-5-aralkyl-1,3,4-thiadiazole (2a–c)

The mixture of substituted/phenyl acetic acid **1** (0.1 M) and thiosemicarbazide (0.15 M) was added slowly to the round bottom flask containing concentrated H_2SO_4 (30 mL) with constant stirring, in ice bath. After complete addition, ice bath was replaced by water bath and slowly heated to 70–80^{o}C and maintained at that temperature for 7 h. After cooling to room temperature, the contents of reaction mixture were poured into ice water and made basic with ammonia, precipitate was filtered, washed with water and recrystallized from ethanol.

2-Amino-5-benzyl-1,3,4-thiadiazole (**2a**) (19)

Yield: 55%, m.p. 201-203^{o}C. IR (cm^{-1}): 3021, 2936, 1520, 1465, 1350, 1300, 1172, 1095, 1010; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.14 (s, 2H, -CH_2-), 7.12 (s, -NH_2, 2H), 7.23–7.34 (m, 5H). MS EI (m/z): 192.00 (M+1).

2-Amino-5-(4-chlorobenzyl)-1,3,4-thiadiazole (**2b**) (20)

Yield: 55%, m.p. 181-182^{o}C. IR (cm^{-1}): 3262, 3100, 2971, 2915, 1698, 1519, 1491, 1332, 1091, 1016; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.30 (s, 2H, -CH_2-), 7.06 (s, 2H, -NH_2), 7.55 (d, *J* = 8 Hz, 2H), 8.20 (d, *J* = 8 Hz, 2H). MS EI (m/z): 226.10 (M+1).

2-Amino-5-(4-methylbenzyl)-1,3,4-thiadiazole (**2c**) (20)

Yield: 52 %, m.p. 208-212^{o}C. IR (cm^{-1}): 3396, 3270, 3126, 2922, 1605, 1516, 1430, 1342, 1147, 1048; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.27 (s, 3H, CH_3), 4.08 (s, 2H. -CH_2-), 7.03 (s, 2H, -NH_2), 7.14 (s, 4H, arom.).

General method for synthesis of 2-aralkyl-6-arylimidazo[2,1-*b*][1,3,4] thiadiazole (3a-3l)

A mixture of 2-amino-5-aralkyl-1,3,4-thiadiazole **2** (0.02 M) and appropriate phenacyl bromide (0.02 M) in ethyl alcohol (50 mL) was refluxed on water bath for 10-12 h. An excess of solvent was removed under reduced pressure and the solid hydrobromide that separated was filtered, washed with cold ethanol and dried. Neutralization of the above hydrobromide salts were done with cold aqueous solution of sodium carbonate (pH 7) to get corresponding bases. All free bases were purified by recrystallization from ethyl alcohol.

2-Benzyl-6-(4-chlorophenyl)imidazo[2,1-*b*][1,3,4] thiadiazole (**3a**) (21)

Yield: 45%, m.p. 163-165^{o}C. IR (cm^{-1}): 3127, 3061, 3032, 2917, 2848, 1523, 1256, 1027; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.44 (s, 2H, -CH_2-), 7.23–7.49 (m, 7H, ar), 7.82 (s, 1H, ar), 8.73 (s, 1H, im). MS (m/z): 326.10 (M). Analysis: calcd. for $C_{17}H_{12}ClN_3S$: C 62.67; H 3.71; N 12.90%; found: C 62.45; H 3.65; N 12.98%. R_f: 0.78.

2-Benzyl-6-(4-nitrophenyl)imidazo[2,1-*b*][1,3,4] thiadiazole (**3b**) (21)

Yield: 55%, m.p. 219-222^{o}C. IR (cm^{-1}): 3132, 3074, 2931, 2830, 1600, 1504, 1469, 1339, 1029; ^{1}H NMR (400 MHz, DMSO-d_6, δ. ppm): 4.46 (s, 2H, -CH_2-), 7.29–7.42 (m, 5H), 8.10 (d, *J* = 8 Hz, 2H), 8.27 (d, *J* = 8 Hz, 2H), 8.92 (s, 1H, im). MS (m/z): 337.00 (M+1). Analysis: calcd. for $C_{17}H_{12}N_4SO_2$: C 60.70; H 3.60; N 16.66%; found: C: 60.15; H: 3.49; N: 16.94%. R_f: 0.63.

2-Benzyl-6-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3c**) (21)

Yield: 48%, m.p. 160-162^{o}C. IR (cm^{-1}): 3134, 3058, 3033, 2973, 2864, 1524, 1470, 1232, 1177, 1092; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.30 (s, 3H, -CH_3), 4.43 (s, 2H, -CH_2-), 7.20 (d, *J* = 8 Hz, 2H), 7.29–7.41 (m, 5H), 7.73 (d, *J* = 8 Hz, 2H), 8.55 (s, 1H, im). MS (m/z): 306.00 (M+1). Analysis: calcd. for $C_{18}H_{15}N_3S$: C 70.79; H 4.95; N 13.76%; found: C 70.85; H 4.52; N 13.91%. R_f: 0.72.

2-Benzyl-6-(4-methoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3d**) (21)

Yield: 51%, m.p. 180-182^{o}C. IR (cm^{-1}): 3133, 3062, 2960, 2910, 1611, 1522, 1488, 1430, 1244; ^{1}H NMR (400 MHz, DMSO-d_6, δ. ppm): 3.84 (s, 3H, -OCH_3), 4.33 (s, 2H, -CH_2-), 6.97 (d, *J* = 8 Hz, 2H), 7.31-7.41 (m, 5H), 7.74 (d, *J* = 8 Hz, 2H), 7.88 (s, 1H, im). MS (m/z): 322.10 (M+1). Analysis: calcd. for $C_{18}H_{15}N_3OS$: C 67.27; H 4.70; N 13.07%; found: C 66.98; H 4.59; N 13.25%. R_f: 0.68.

2-(4-Chlorobenzyl)-6-(4-chlorophenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3e**) (21)

Yield: 52%, m.p. 175-178^{o}C. IR (cm^{-1}): 3147, 3057, 2927, 2861, 1528, 1476, 1403, 1092; ^{1}H NMR

(400 MHz, DMSO-d_6, δ, ppm): 4.27 (s, 2H, -CH_2-), 7.27 (d, *J* = 8 Hz, 2H), 7.35–7.38 (dd, *J* = 8, 8 Hz, 4H), 7.72 (d, *J* = 8 Hz, 2H), 7.94 (s, 1H, im). MS (m/z): 360.00 (M). Analysis: calcd. for $C_{17}H_{11}Cl_2N_3S$: C 56.68; H 3.08; N 11.66%; found: C 56.75; H 3.01; N 11.71%. R_f: 0.73.

2-(4-Chlorobenzyl)-6-(4-nitrophenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3f**) (21)

Yield: 55%, m.p. 195-198°C. IR (cm^{-1}): 3130, 2925, 2832, 1597, 1504, 1414, 1338, 1266, 1098; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 4.47 (s, 2H, -CH_2-), 7.44 (m, 4H), 8.09 (d, *J* = 8 Hz, 2H), 8.26 (d, *J* = 8 Hz, 2H), 8.91 (s, 1H, im). MS (m/z): 369.00 (M-2). Analysis: calcd. for $C_{17}H_{11}ClO_2N_4S$: C 55.06; H 2.99; N 15.11%; found: C 54.98; H 3.01; N 15.19%. R_f: 0.67.

2-(4-Chlorobenzyl)-6-(4-methylphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3g**) (21)

Yield: 50%, m.p. 178-181°C. IR (cm^{-1}): 3143, 3055, 3021, 2961, 2924, 2865, 1596, 1535, 1461, 1290, 1935; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.37 (s, 3H, -CH_3), 4.26 (s, 2H, -CH_2-), 7.22 (d, *J* = 8 Hz. 2H), 7.27 (d, *J* = 8 Hz, 2H), 7.36 (d, *J* = 8 Hz, 2H), 7.69 (d, *J* = 8 Hz, 2H), 7.92 (s, 1H, im). MS (m/z): 340.00 (M). Analysis: calcd. for $C_{18}H_{14}ClN_3S$: C 63.62; H 4.15; N 12.36%; found: C 63.75; H 4.01; N 12.41%. R_f: 0.69.

2-(4-Chlorobenzyl)-6-(4-methoxyphenyl)imidazo[2,1-*b*][1,3,4]thiadiazole (**3h**) (21)

Yield: 60%, m.p. 174-176°C. IR (cm^{-1}): 3146, 3040, 2939, 2842, 1609, 1537, 1482, 1252, 1025; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 3.84 (s, 3H, -OCH_3), 4.96 (s, 2H, -CH_2-), 6.96 (d, *J* = 8 Hz, 2H), 7.27 (d, *J* = 8 Hz, 2H), 7.36 (d, *J* = 8 Hz, 2H), 7.73 (d, *J* = 8 Hz, 2H), 7.88 (s, 1H, im). MS (m/z): 356.00 (M). Analysis: calcd. for $C_{18}H_{14}ClN_3OS$: C 68.03; H 5.11; N 12.53%; found: C 68.15; H 5.05; N 12.75%. R_f: 0.71.

2-(4-Methylbenzyl)-6-(4-chlorophenyl)-imidazo[2,1-*b*][1,3,4]thiadiazole (**3i**)

Yield: 50%, m.p. 226-228°C. IR (cm^{-1}): 3124, 2909, 1525, 1470; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.83 (s, 3H, -CH_3), 4.37 (s, 2H, -CH_2-), 7.18 (d, *J* = 8 Hz, 2H), 7.28 (d, *J* = 8 Hz, 2H), 7.44 (d, *J* = 8 Hz, 2H), 7.86 (d, *J* = 8 Hz, 2H), 8.66 (s, 1H, im). MS (m/z): 362.10 (M+Na). Analysis: calcd. for $C_{18}H_{14}ClN_3S$: C 63.62; H 4.15; N 12.36%; found: C 63.55; H 4.10; N 12.41%. R_f: 0.73.

2-(4-Methylbenzyl)-6-(4-nitrophenyl)-imidazo[2,1-*b*][1,3,4]thiadiazole (**3j**)

Yield: 45%, m.p. 170-175°C. IR (cm^{-1}): 3125, 2867, 1598, 1414; Analysis: calcd. for $C_{18}H_{14}N_4O_2S$: C 61.70; H 4.03; N 15.99%; found: C 61.59; H 3.99; N 16.07%. R_f: 0.72.

Table 1. Inhibition of paw edema in rats by synthesized compounds.

Compd.	Inhibition (%)	
	At 3rd h	At 5th h
3a	26.34 ± 0.89	78.87 ± 3.80***b
3b	14.87 ± 4.64	63.10 ± 3.24
3c	49.99 ± 0.00***a	34.17 ± 5.58‡
3d	41.65 ± 5.27 *a	75.00 ± 3.73
3e	37.50 ± 12.50	83.33 ± 0.00
3f	17.49 ± 2.50	55.00 ± 3.42
3g	30.35 ± 1.44	52.38 ± 6.82
3h	20.83 ± 2.38	59.45 ± 5.60
3i	19.99 ± 2.23	56.11 ± 3.89
3j	22.21 ± 4.65	63.89 ± 9.51
3k	36.66 ± 6.01	51.11 ± 4.36†
3l	36.66 ± 6.01	51.11 ± 4.36†
Ibuprofen	14.16 ± 1.89	71.67 ± 3.80

Values are the mean ± SEM, n = 6. ***b - $p < 0.01$, when compared to ibuprofen at 5th hour. ***a - $p < 0.001$, when compared to ibuprofen at 3rd hour. *a - $p < 0.05$, when compared to ibuprofen at 3rd hour. ‡ - $p < 0.01$, when compared to ibuprofen at 5th hour. † - $p < 0.05$, when compared to ibuprofen at 5th hour.

2-(4-Methylbenzyl)-6-(4-methylphenyl)-imidazo[2,1-*b*][1,3,4]thiadiazole (3k)

Yield: 52%, m.p. 180-182°C. IR (cm^{-1}): 3118, 2868, 1587, 1460; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.28 (s, 3H, -CH_3), 2.30 (s, 3H, -CH_3), 4.37 (s, 2H, -CH_2-), 7.19 (d, *J* = 8 Hz, 2H), 7.20 (d, *J* = 8 Hz, 2H), 7.28 (d, *J* = 8 Hz, 2H), 7.73 (d, *J* = 8 Hz, 2H), 8.54 (s, 1H, -CH-). MS (m/z): Analysis: calcd. for $C_{19}H_{17}N_3S$: C 71.44; H 5.36; N 13.15%; found: C 71.06; H 5.25; N 13.21%. R_f: 0.65.

2-(4-Methylbenzyl)-6-(4-methoxyphenyl)-imidazo[2,1-*b*][1,3,4]thiadiazole (3l)

Yield: 55%, m.p. 138-140°C. IR (cm^{-1}): 3141, 2936, 1603, 1477; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 2.28 (s, 3H, -CH_3), 3.76 (s, 3H, -OCH_3), 4.36 (s, 2H, -CH_2-), 6.94-7.78 (m, 8H, ar), 8.48 (s, 1H, -CH-). MS (m/z): 358.10 (M+Na). Analysis: calcd. for $C_{19}H_{17}N_3OS$: C 68.03; H 5.11; N 12.53%; found: C 67.95; H 5.01; N 12.61%. R_f: 0.68.

Antiinflammatory activity

Acute antiinflammatory activity of selected compounds was evaluated in Wistar albino rats, after taking clearance for animal experimentation from Institutional Animal Ethics Committee of KLE University's College of Pharmacy, Bangalore. Naive animals (200-250 g, either sex) were challenged with 0.5 mL of 1% carrageenan [intraplantar injection into right paw] (22), after one hour of oral administration of newly synthesized compounds in the dose of 100 mg/kg. Simultaneously, a group of animals was treated with ibuprofen (100 mg/kg body weight) for comparative purposes. Edema, a cardinal sign of inflammation, was measured at regular intervals (3rd, 5th h, post challenge) by mercury displacement method using plethysmograph (23). Percentage inhibition of edema in various groups of animals treated with newly synthesized compounds and ibuprofen are tabulated in Table 1.

Statistical analysis

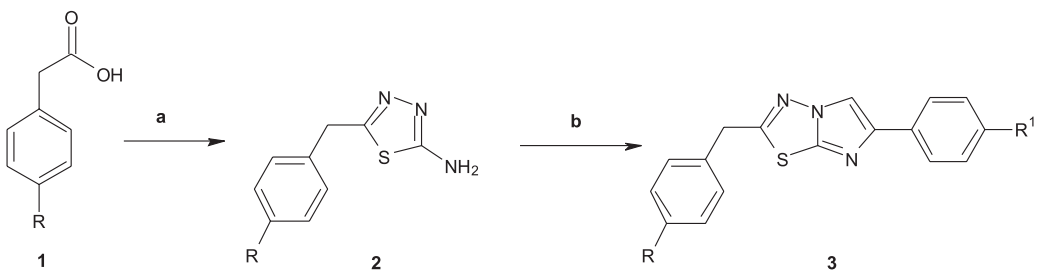
Statistical significance for inhibition of edema between various groups was determined using one way ANOVA followed by *post-hoc* Newman-Keuls multiple comparison. Values $p < 0.05$ was considered significant.

RESULT AND DISCUSSION

Chemistry

We have synthesized a series of 12 derivatives of imidazo[2,1-*b*][1,3,4]thiadiazoles containing aralkyl group at 2nd position by reacting 2-amino-5-aralkyl-1,3,4-thiadiazoles **1** with 4-substituted phenacyl bromide (Scheme 1). Structures of the synthesized compounds were established on the basis of IR, ^{1}H NMR and MS analysis. All synthesized compounds showed absorption bands ranging from 3141 to 3118 cm^{-1} for C-H aromatic stretching and 2936 to 2868 cm^{-1} for C-H aliphatic stretching.

In ^{1}H NMR, the presence of singlet between δ 8.66-8.48 ppm for imidazole proton (C5-H) confirmed the cyclization of compound **2** with 4-substi-



R = H (**a**); Cl (**b**); CH_3 (**c**)

R = H, R^1 = Cl (**a**); R = H, R^1 = NO_2 (**b**);
R = H, R^1 = CH_3 (**c**); R = H, R^1 = OCH_3 (**d**)
R = Cl, R^1 = Cl (**e**); R = Cl, R^1 = NO_2 (**f**);
R = Cl, R^1 = CH_3 (**g**); R = Cl, R^1 = OCH_3 (**h**);
R = CH_3, R^1 = Cl (**i**); R = CH_3, R^1 = NO_2 (**j**);
R = CH_3, R^1 = CH_3 (**k**); R = CH_3, R^1 = OCH_3 (**l**)

Scheme 1. Synthesis of compounds **3a**-**3l**. Reagents and conditions: a - thiosemicarbazide, H_2SO_4, NH_3; b - 4-substituted phenacyl bromides, ethanol, reflux, Na_2CO_3

tuted phenacyl bromide. Compounds (**3a-3l**) showed prominent signals for aromatic protons around δ 7.86-6.94 ppm. Methylene proton at C_2 appeared between δ 4.37-4.36 ppm for synthesized derivatives (**3a-3l**). Compounds **3a**, **3l**, **3k** and **3j** showed singlet between δ 2.83-2.28 ppm for the presence of methyl proton on phenyl ring. Compound **3l** showed a singlet at δ 3.76 ppm for the presence of -OCH_3 at the phenyl ring.

Antiinflammatory activity

Acute antiinflammatory activities of newly synthesized compounds (**3a-3l**) were evaluated by carrageenan induced paw edema of rat at the dose of 100 mg/kg (p. o.) and expressed as percentage of inhibition of edema, after 3 and 5 h as shown in Table 1. By 3rd hour, percentage of edema inhibition ranged between 14 and 47% and by 5th hour, between 34 and 78%, suggestive of prolonged anti-inflammatory activity.

By 3rd hour, all compounds demonstrated anti-inflammatory activity similar or higher than that of standard NSAID, ibuprofen. However, **3c** and **3d** demonstrated significantly ($p < 0.001$ and $p < 0.05$, respectively) higher activity than ibuprofen. Even after 5 hours, all compounds continue to demonstrate significant antiinflammatory activity (except for **3c** and **3k** and **3l**) where the activity was significantly reduced compared to ibuprofen. Animals treated with **3a** recorded a higher and significant ($p < 0.001$) antiinflammatory activity than ibuprofen by 3rd hour of post-carrageenan challenge.

Structure activity relation

Imidazo[2,1-*b*][1,3,4]thiadiazole derivatives with substituents like Cl, NO_2, CH_3 and OCH_3 at 4th position of phenyl group did not gave any improvement in antiinflammatory activity in comparison to ibuprofen by 3rd hour treatment except derivatives **3b**, **3f** and **3i**. However, when substitutions were made at 4th position of both benzyl and phenyl groups of imidazo[2,1-*b*][1,3,4] thiadiazole, by electron withdrawing and electron donating groups, compounds demonstrated significant antiinflammatory activity in the dose of 100 mg/kg body weight.

CONCLUSION

We have synthesized 12 derivatives of 2,6-disubstituted imidazo[2,1-*b*][1,3,4]thiadiazoles by reacting 2-amino-5-aralkyl-1,3,4-thiadiazole with various phenacyl bromides in good yields. Among the tested compounds, compound **3c** with the 4-methyl substitution in the phenyl ring at 6 position of the imidazo[2,1-*b*][1,3,4] thiadiazole ring demonstrated 34% edema inhibition after 5th hour of carrageenan challenge. Further studies are required to establish its exact mechanism of action.

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SYNTHESIS, CHARACTERIZATION, ANTIMICROBIAL AND PHYTOTOXIC SCREENING OF 1-AROYL-3,5-DIARYLPYRAZOLINE DERIVATIVES

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Abstract: Pyrazolines are biologically and pharmaceutically very active scaffolds. Derivatives of (3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl)(phenyl)methanone were synthesized by the cyclization of chalcones (**1a-c**) with substituted benzyl hydrazides (**2a-e**) using a few drops of piperidine as catalyst. Structures of all the synthesized compounds were confirmed by FTIR, ^{1}H NMR, ^{13}C NMR and mass spectrometric analysis. All the pyrazolines were subjected to antimicrobial and phytotoxic assays. Compound **3a** and **3c** showed maximum antimicrobial activities while all the synthesized compounds were active acc. to their phytotoxic assays.

Keywords: antimicrobial, chalcones, phytotoxic, pyrazolines

With an increase in the resistance of the microorganisms to the existing drugs, a need for the development of new synthetic routes for the synthesis of biologically and industrially significant compounds is increasing day by day (1). In this regard, organic synthesis is playing a significant role in the development of new molecules with broad spectrum of activities (2). Due to significant contribution of heterocyclic compounds for the treatment of many infectious diseases they have attained a special attention of many scientists. With the increase in world population, a need for the improvement in food growth is also increasing. Heterocycles are also playing an important role in the field of insecticides and pesticides synthesis (3-5). Among them pyrazolines derivatives are pharmacologically active compounds with a broad spectrum of activities including antimicrobial (6), antioxidant, anticancerous (7), anti hypertensive, antifungal, and malarial (8) insecticidal, (9) herbicidal and anti-inflammatory activities (7, 10-12).

With all this significant importance, we have synthesized some 1-aroyl-3,5-diarylpyrazolines under standard reaction conditions (13).

RESULTS AND DISCUSSION

Derivatives of (3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl)(phenyl)methanone were synthesized by the reaction of substituted hydrazides (**2a**-**e**) with substituted chalcones (**1a**-**c**) in the presence of piperidine as a catalyst in dry ethanol (13, 14). All the synthesized compounds were subjected to determination of antimicrobial and antifungal activities using agar well diffusion method (with concentration of 5 mg/mL) and disc diffusion method (with the same concentration), respectively. Phytotoxic activities were carried out on the surface of the broad leaves of Physalis plant. In the FTIR spectral data, stretching for pyrazoline ring was observed at 2800-2934 cm^{-1} (15). ^{1}H NMR spectra structures were confirmed due to the presence of a stereogenic center at 5 position of the pyrazoline ring, three characteristic peaks were observed for 4Ha, 4Hb and 5H protons. A doublet of doublets was observed at δ 5.2-5.4 ppm (J = 10.1, 5.5 Hz) for 5H proton and also at δ 4.1-4.25 (11.9, 9.7 Hz) ppm and δ 3.9-4.0 (10.2, 4.1 Hz) ppm for 4Ha and 4Hb protons of the pyrazoline ring, respectively (16, 17) . In ^{13}C NMR spectra, signal for C-5 carbon at δ 59-62 ppm and that for C-4 carbon at δ 43 ppm were observed (8, 18, 19). In mass fragmentation pattern of pyrazolines, the molecular ion peak and base peak derived from benzoyl group were observed. In the antibacterial bioassay, maximum inhibition was observed by compounds **3a** and **3c** with two methoxy electron donating groups at 3 and 5 positions of ring 'A' and a para chloro group on ring 'C' of the pyrazoline. In

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case of antifungal activities, the maximum percentage inhibition was observed for the same compounds, while all other pyrazolines showed moderate inhibition against yeast cells. From above observations it is found that molecules with methoxy and chloro groups shows more inhibition as compared to the others (20). In the case of phytotoxic activities, pale yellowing of the leaves were observed, which is due to binding of the compound with the chemoreceptor of the Physalis plants leaves (21). During phytotoxic assay of pyrazolines, almost all the compounds could actively bind with the chemoreceptor and have the potential to inhibit the growth of plant by ceasing photosynthesis (Table 1)

EXPERIMENTAL

Melting points were recorded using a digital Gallenkamp (SANYO) model MPD BM 3.5 apparatus and are uncorrected. ^{1}H NMR spectra were determined for $CDCl_3$ solutions at 300 MHz and ^{13}C NMR spectra were recorded for the same solutions at 75 MHz using a Bruker AM-300 spectrophotometer. FTIR spectra were recorded using an FTS 3000 MX spectrophotometer; mass spectra (EI, 70 eV) were recorded on a GC-MS instrument, Agilent Technology USA. Rf values were deretmined for mobile phase: petroleum ether : ethyl acetate (3 : 2, v/v). All compounds were purified by thin layer chromatography using silica gel from Merck (Germany).

General procedure for the synthesis of 1-aroyl-3,5-disubstituted pyrazolines (3a-h)

Ethanolic solution of appropriate chalcone (**1a-c**, 0.5 mol) and hydrazide (**2a-e**, 0.5 mol) were refluxed for two hours in the presence of few drops of piperidine as catalyst. Completion of reaction was confirmed by TLC. Upon completion, the resulting mixture was concentrated and purified by using precoated TLC chromatography (13).

(±)-(5-(4-Chlorophenyl)-3-(2-hydroxy-5-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dimethoxyphenyl)methanone (3a)

Yield: 92%; R_f 0.9; m.p. 117-118^{o}C. IR (KBr, cm^{-1}): 3315, 2932, 2812, 1712, 1621, 1596, 1437, 1243; ^{1}H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 10H arom.), 5.2 (dd, 1H, *J* = 9.0, 3.9 Hz, H5), 4.4 (dd, 1H, *J* = 5.8, 3.1 Hz, H4a), 4.2 (dd, 1H, *J* = 6.1, 3.3 Hz, H4b), 3.9 (s , 6H), 1.6 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 168 (CO), 162 (C-3), 61 (C-5), 43 (C-4). EIMS (m/z, %): 450 [M^{+}] (12), 452 (4), 285 (10), 165 (100), 133 (42).

(±)-(5-(4-Chlorophenyl)-3-(2-hydroxy-5-methylphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,4,5-trimethoxyphenyl)methanone (3b)

Table 1. Antimicrobial and phytotoxic activities of compounds **3a-h**.

Compound	Antibacterial		Antifungal *S. cerevisiae*		Phytotoxic activity
	E. coli	*B. subtilis*	(ZI)	(PI)	
3a	7	16	0.4	66.66	Pale yellow
3b	8	11	0.5	58.33	Pale yellow
3c	6	16	0.4	66.6	Pale yellow
3d	9	14	0.8	33.3	Pale yellow
3e	7	12	0.9	25.0	Pale yellow
3f	12	14	0.5	58.33	Pale yellow
3g	5	13	0.5	58.3	Pale yellow
3h	9	15	0.4	66.6	Pale yellow
Negative control (acetone)	-	-	-	-	-
Kanamycin	-	20	-	-	-
Ampicillin	15	-	-	-	-
Fluconazone	-	-	12	100	-

Concentration used: 5 mg/mL; -: no activity, ZI (zone of inhibition, radius, mm), PI: percent inhibition
PI = 100 - fungal growth in sample (cm) / fungal growth in control (cm) × 100.

Yield: 85%; R_f 0.9; m.p. 101-102°C. IR (KBr, cm^{-1}): 3311, 2965, 2913, 1713, 1622, 1583, 1431, 1257. ^1H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 12H arom.), 5.2 (dd, 1H, *J* = 9.6, 3.9 Hz, H5), 4.3 (dd, 1H, *J* = 5.7, 3.3 Hz, H4a,), 4.2 (dd, 1H, *J* = 6.1, 3.3 Hz, H4b), 3.9 (s , 9H), 1.6 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 167 (CO), 163 (C-3), 59 (C-5), 43 (C-4). EIMS (m/z, %): 460 (21), 462 (5) [M^+], 265 (34), 195 (100), 133 (56).

(±)-(5-(4-Chlorophenyl)-3-(2-hydroxy-5-methylphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,5-dimethoxy-4-methylphenyl)methanone (3c)

Yield: 78%; R_f 0.9; m.p. 141-142°C. IR (KBr, cm^{-1}): 3017, 2965, 2911, 1707, 1621, 1597, 1433, 1257 cm^{-1}; ^1H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 12H arom.), 5.4 (dd, 1H, *J* = 9.6, 3.3, H5,), 4.2 (dd, 1H, *J* = 5.7, 3.3, H4a), 4.1 (dd, 1H, *J* = 5.7, 3.3, H4b), 3.9 (s , 6H), 2.348 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 170 (CO), 161 (C-3), 58 (C-5), 43 (C-4). EIMS (m/z, %): 462 (37), 464 (53) [M^+], 285 (21), 179 (100), 133 (49).

(±)-(2E,4E)-1-(5-(4-chlorophenyl)-3-(2-hydroxy-5-methylphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-5-phenylpenta-2,4-dien-1-one (3d)

Yield: 85%; R_f 0.9; semisolid. IR (KBr, cm^{-1}): 3012, 2966, 2917, 1722, 1665, 1571, 1433; ^1H NMR ($CDCl_3$, δ, ppm): 6.6-7.30 (m, 12H arom.), 7.4 (d, 1H, *J* = 6.3 Hz, $H_ß$), 7.1 (d, 1H, *J* = 3.4 Hz, $H_α$), 6.9 (d, 1H, *J* = 4.2 Hz, $H_γ$), 6.6 (d, 1H, *J* = 3.6 Hz, $H_δ$), 5.4 (dd, 1H, *J* = 9.6, 3.3 Hz, H5), 4.1 (dd, 1H *J* =5.7, 3.3 Hz, H4a), 3.9 (dd, 1H, *J* = 5.7, 3.3 Hz, H4b), 2.5 (s, 3H, CH3); ^{13}C NMR ($CDCl_3$, δ, ppm): 166 (CO), 157 (C-3), 61 (C-5), 43 (C-4). EIMS (m/z, %): 442 (21), 444 (11) [M^+], 133 (47), 90(100).

(±)-(3-(2-Hydroxy-5-methylphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)(3,4,5-trimethoxyphenyl)methanone (3e)

Yield: 80%; R_f 0.8; m.p. 170-171°C. IR (KBr, cm^{-1}): 3121, 2964, 2911, 1706, 1675, 1595, 1431; ^1H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 9H arom.), 5.1 (dd, 1H, *J* = 9.0, 3.3 Hz, H5), 4.24 (dd, 1H, *J* = 5.7, 3.6 Hz, H4a), 4.1 (dd, 1H, *J* = 5.7, 3.6 Hz, H4b), 3.9 (s, 12H, OCH3), 1.6 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 167 (CO), 162 (C-3), 60 (C-5), 43 (C-4); EIMS (m/z, %): 540 (11), 542 (32) [M^{+o}], 345 (12), 196 (10), 195 (100).

(±) (3,5-Dimethoxy-4-methylphenyl)(3-(2-hydroxy-5-methylphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)methanone (3f)

Yield: 83%; R_f 0.6; oil. IR (KBr, cm^{-1}): 3021, 2963, 2943, 1700, 1686, 1595, 1435; ^1H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 9H arom.), 5.1 (dd, 1H, *J* = 9.0, 3.3 Hz, H5), 4.24 (dd, 1H, *J* = 5.7, 3.6 Hz, H4a), 4.1 (dd, 1H, *J* = 5.7, 3.6 Hz, H4b), 3.9 (s, 9H, OCH_3), 2.52 (s, 3H, CH3), 1.6 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 168 (CO), 161 (C-3), 59 (C-5), 43 (C-4). EIMS (m/z, %): 524 (11), 526 (25) [M^+], 345 (21), 196 (27), 179 (100).

(±)-(3,5-Dimethoxyphenyl)[3-(2-hydroxy-5-methylphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]methanone (3g)

Yield: 78%; R_f 0.8; m.p. 160-161°C. IR (KBr, cm^{-1}): 3021, 2963, 2919, 1742, 1656, 1595,1441; ^1H NMR ($CDCl_3$, δ, ppm): 6.9-7.7 (m, 10H arom.), 5.1 (dd, 1H, *J* = 9.0, 3.3 Hz, H5), 4.2 (dd, 1H, *J* = 5.1, 3.6 Hz, H4a), 4.0 (dd, 1H, *J* = 5.1, 3.6 Hz, H4a), 3.9 (s, 9H, OCH_3), 1.6 (s, 3H); ^{13}C NMR ($CDCl_3$, δ, ppm): 168 (CO), 162 (C-3), 61 (C-5), 43 (C-4). EIMS (m/z, %): 510, 512 [M^+] (32), 345 (11), 196 (29), 165 (100).

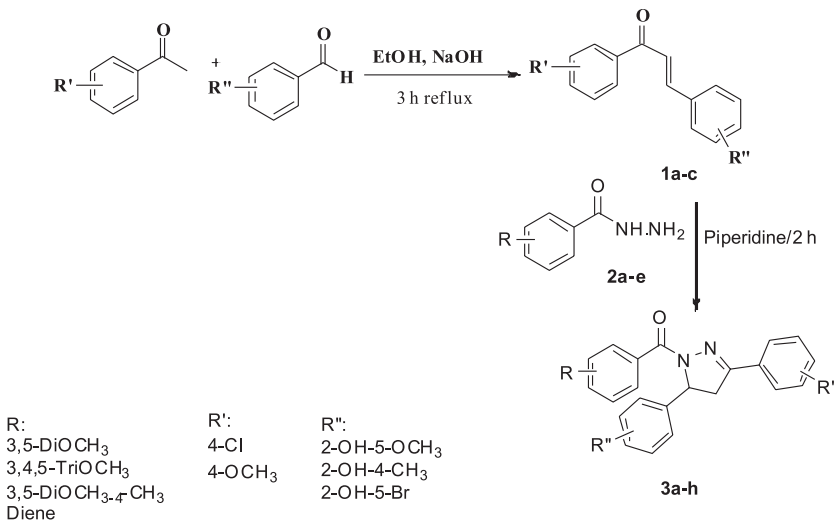
(±) (2E,4E)-1-[3-(5-bromo-2-hydroxyphenyl)-5-(4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl]-5-phenylpenta-2,4-dien-1-one (3h)

Yield: 82%; R_f 0.9; oil. IR (KBr, cm^{-1}): 3023, 2961, 2920, 1698, 1656, 1595,1441; ^1H NMR ($CDCl_3$, δ, ppm): 6.6-7.30 (m, 12H arom.), 7.4 (d, 1H, *J* = 6.3 Hz, $H_ß$), 7.1 (d, 1H, *J* = 3.4 Hz, $H_α$,), 6.9 (d, 1H, *J* = 4.2 Hz, $H_γ$), 6.6 (d, 1H, *J* = 3.6 Hz, $H_δ$), 5.4 (dd, *J* = 9.6, 3.3 Hz, H5), 4.1 (dd, *J* = 5.7, 3.3 Hz, H4a), 3.9 (dd, *J* = 5.7, 3.3 Hz, H4b); ^{13}C NMR ($CDCl_3$, δ, ppm): 168 (CO), 162 (C-3), 61 (C-5), 43 (C-4); EIMS (m/z, %): 502, 504 [M^{+o}] (19), 198 (23), 90 (100).

Biological assay

Antibacterial activities

Antibacterial *in vitro* bioassays were conducted by using agar well diffusion method (22) against Gram-positive (*Bacillus subtilus*) and Gram-negative (*Escherichia coli*) strains of bacteria, ampicillin (against Gram-negative) and kanamycin (against Gram-positive) were used as standards. For analysis, concentrations of 3 mg/mL and 5 mg/mL of compounds dissolved in 1 mL of acetone were used. Experiments were carried out on Mueller-Hinton plates. Laboratory strain bacteria (*Bacillus subtilis*, *Escherichia coli*) were grown to log phase in LB (1% yeast extract, 1% peptone and 1% dextrose) at 37°C for overnight with constant shaking. Cultures were spread onto the plates, then wells were made by using cork borer (4 mm). Wells were loaded with: 3 mg/mL and 5 mg/mL solution of compounds in acetone, negative control acetone and positive



Scheme 1. Synthesis of 1-aroyl-3,5-diarylpyrazoline derivatives

control ampicillin and kanamycin with the help of 5 µL micropipette. Plates were incubated for 24 h at 37°C. Tests were repeated three times. Most significant results were obtained using a concentration of 5 mg/mL. After 24 h incubation time, the zones of inhibition were evaluated in mm scale and compared with standard drugs (22).

Antifungal activities

Antifungal activities were carried out against yeast (*Saccharomyces cerevisiae*). Fluconazole was used as standard drug. Concentrations of 3 mg/mL and 5 mg/mL of compounds were used for the antifungal analysis. For antifungal activities, plates were loaded with sample solutions, standard drug and negative control acetone with the help of 5 µL micropipette and incubated for 24 h at 28°C. Tests were repeated three times. Concentration of 5 mg/mL showed most significant results. After 24 h incubation time, the zones of inhibition were examined and percentage of fungal inhibition was calculated and compared with reference to the standard drugs (23) according to equation:.

% of fungal inhibition = 100 - fungal growth in test sample (cm) / fungal growth in control (cm) ×100.

Phytotoxic activities

All the synthesized compounds were tested for their phytotoxic activities as an effect of chemicals on the growth inhibition of plant. Leaves of Physalis plant (the fruit of this plant is called Cape gooseberry used as a decorative of cakes) were used for the above test. Solutions of compounds (3 and 5 mg/mL) were applied on the broad leaves of the Physalis plants. Plants were left for 24 h, then phytotoxic affects of the compounds were observed by blackening or pale yellowing of the leave (21).

CONCLUSION

Successful synthesis of the derivatives of (3,5-diphenyl-4,5-dihydro-1H-pyrazol-1-yl)(phenyl)-methanone were carried out. Structures of all the synthesized compounds were confirmed by IR, ^{1}H NMR, ^{13}C NMR and mass spectrometric analysis. All the compounds were subjected to their antibacterial, antifungal and phytotoxic assays. Among them, compounds **3a** and **3c** showed maximum inhibition against the bacterial and fungal strains while all compound were active for their phytotoxic activities.

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF NOVEL N,N-CYCLIC-2,4-DIHYDROXYTHIOBENZAMIDE DERIVATIVES*

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Abstract: A series of novel N,N-cyclic-2,4-dihydroxythiobenzamide derivatives is described. Test compounds were formed by the reaction of the commercially available reagents with sulfinylbis[(2,4-dihydroxyphenyl)methanethione] (STB). The chemical structures were confirmed by IR, ^{1}H NMR, ^{13}CNMR, EI-MS, and elemental analysis. For the estimation of potential activity *in vitro*, the MIC values against strains of *Candida* were determined. Antifungal properties of selected compounds under *in vitro* conditions against five phytopathogenic fungi were estimated. Furthermore, the antiproliferative activity against the HCV29T cancer cell lines has been studied. These compounds exhibited antiproliferative activity in the range of 33.98 – 10.51 μg/mL.

Keywords: 2,4-dihydroxythiobenzamide, synthesis, antifungal activity, antiproliferative activity

Cancer is a major health problem in developed countries where it is the second cause of death mainly associated with ageing of the population and lifestyle. Early diagnosis, common access to health care and developed therapies have resulted in a significant improvement of cancer survival (1). Most often, it is chemotherapy that is used in the treatment of cancer. It disrupts the way cancer cells grow and divide. However, it also affects normal cells. Chemotherapy uses cytotoxic drugs to destroy cancer cells and is used alone to treat some types of cancer. Nevertheless, it is frequently used with other treatments such as surgery, radiotherapy, hormonal therapy or other anti-cancer drugs, as targeted or biological therapies. There are many different chemotherapy drugs and new drugs are being developed all the time (2).

An important problem in the chemotherapy not solved yet is treatment of systemic mycoses. There is an increased risk of oral fungal infection during cancer therapy. Some of fungal organisms, notably *Candida albicans*, are commensal inhabitants of the oral cavity. Under normal conditions, these fungal organisms co-exist with the other microorganisms of the normal oral flora and do not cause disease. However, changes in the oral and systemic environment can result in an overgrowth of these fungal species, leading to oral fungal infection. These changes include immunosuppression (e.g., induced by drugs), imbalance in the oral flora (secondary to antibiotic therapy) and local tissue damage (chemotherapy and radiation therapy). Cancer patients receiving chemotherapy and/or radiation therapy are prone to all of the aforementioned predisposing factors and are therefore considered to be at higher risk for oral fungal infection than the general population. Systemic antifungals are poorly effective in the prevention of oral fungal infection in patients receiving cancer therapy. Similarly, currently available topical antifungal agents are less efficacious, suggesting a need for better topical agents (3, 4).

Fungicides remain vital for effective control of plant diseases, which are estimated to cause yield reductions by almost 20% in the major food crops worldwide. During the past few years, fungicide research has produced a diverse range of products (e.g., anilinopyrimidines, phenoxyquinolines, oxazolidinediones, spiroketalamines) with novel modes

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of action, which are expected to have a significant impact on disease control (5). Recently, many sulfur analogues with linear =NC(=S)- or cyclic –S-N=N- groups have been prepared, among others, tolnaftate and tolciclate (thiocarbamates) (6).

2,4-Dihydroxyphenyl derivatives of various groups of compounds with >N-C(<)S- moiety both in the linear (benzanilides and amidrazones) (7, 8) and in cyclic (4*H*-3,1-benzothiazines) systems prepared in our laboratory (9) exhibit an interesting level of antifungal activity against moulds, dermatophytes, yeast, yeastlike, and phytopathogenic fungi. Therefore, the newly prepared N,N-cyclic-2,4-dihydroxythiobenzamides have been tested for their antifungal properties.

Benzanilide (N-phenylbenzamide) and thiobenzanilide derivatives possess a broad spectrum of biological activities. They have been found to exhibit antimalarial, antibacterial and antifungal properties (10-13). Derivatives of N-(2-hydroxyphenyl)benzamide have been studied for the last few years as the possible metabolites of the antibacterial active benzo[d]oxazole derivatives (14). Benzamides have promising anti-inflammatory and analgesic properties (15, 16) and anti-convulsant activity (17). What is more, novel inhibitors representing a diverse range of chemical scaffolds have been screened as potent and selective inhibitors of plasmodium falciparum dihydroorotate dehydrogenase, making the enzyme a strong candidate for the development of new antimalarial compounds (18). Benzamides have weak cholinesterase inhibitory properties but protect cholinesterase *in vitro* from stronger inhibitors like dichlorvos (19). Some novel benzamides were found to have high affinity for dopamine D3 (20, 21) and have been reported to inhibit the c-Met tyrosine kinase receptor, which is a potentially important target for the treatment of cancer (22, 23).

2,4-Dihydroxybenzanilides obtained by our team are characterized by inhibitory effect against dermatophytes (24), yeasts (25) and phytopathogenic fungi (26).

At present, the synthesis of thiobenzamides with sulfinylbis[(2,4-dihydroxyphenyl)methanethione] (STB) and the secondary cyclic amines has been carried out in our laboratory. The paper presents the preparation of N,N-cyclic-2,4-dihydroxythiobenzamides, their *in vitro* antifungal activity against *Candida* and phytopathogenic fungi. The antiproliferative activity of selected compounds to cell line of human bladder tumor (HCV29T) was also evaluated.

EXPERIMENTAL

Chemistry

The IR spectra were measured with a Perkin-Elmer FT-IR 1725X spectrophotometer (in KBr). The spectra were made in the range of 600-4000 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on Varian Mercury 400 or Bruker DRX 500 spectrometers. Chemical shifts (δ, ppm) were described in relation to tetramethylsilane (TMS). The spectra MS (EI, 70 eV) were recorded using the apparatus AMD-604. Elemental analyses (C, H, N) were performed with the use of Perkin-Elmer 2400 instrument and were found to be in good agreement (± 0.4%) with the calculated values. The melting points (m.p.) were determined using a Büchi B-540 (Flawil, Switzerland) melting point apparatus.

The purity of the compounds was examined by HPLC (Knauer, Berlin, Germany) with a dual pump, a 20 µL simple injection valve and a UV-visible detector (330 nm). The Hypersil Gold C18 (1.9 µm, 100 × 2.1 mm) column was used as the stationary phase. The mobile phase included different contents of MeOH and acetate buffer (pH 4, 20 nM) as the aqueous phase. The flow rate was 0.5 mL/min at room temperature. The retention time of an unretained solute (t_o) was determined by the injection of a small amount of acetone dissolved in water. The log k values for 70% of MeOH (v/v) in the mobile phase are presented. The log k values were calculated as $\log k = \log(t_R - t_o)/t_o$, where: t_R - the retention time of a solute, t_o - the retention time of an unretained solute.

Synthesis of N,N-cyclic-2,4-dihydroxythiobenzamide derivatives

4-(2,4-Dihydroxybenzencarbothiol)piperazine-1-carboxylate ethyl (1)

STB (0.02 mol) and piperazine-1-carboxylate ethyl (Alfa Aesar) (0.02 mol) were added to MeOH (80 mL) and heated to boiling (3 h). The mixture was hot filtered and water (50 mL) was added to the filtrate. The removed compound was washed by water and recrystallized from MeOH (20 mL) and then from benzene (50 mL).

Yield: 76%; m.p.: 195-197°C; log k = -0.401. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.74 (s, 1 H, HO-C2), 9.56 (s 1 H, HO-C4), 7.00 (d, 1 H, J = 8.6 Hz, H-C6), 6.28 (s, 1 H, H-C3), 6.19 (m, 1 H, H-C5), 4.06 (q, 2 H, J = 7.2 Hz, CH_2), 3.71 (m, 4 H, H-C (piperazine)), 3.15 (m, 4 H, H-C (piperazine)), 1.19 (t, 3 H, J = 7.1 Hz, CH_3); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 169.9 (C=S), 159.0 (C=O),

154.5, 151.8, 130.5, 121.3, 106.6, 101.8, 60.9 (CH_2), 50.33 (C piperazine), 48.5 (C piperazine), 43.2 (C piperazine), 40.0 (C piperazine), 14.4 (CH_3); IR (KBr, cm^{-1}): 3220 (OH), 1669 (C=O), 1613 (C=C), 1594 (C=C), 1493, 1435 (C-H), 1386, 1350, 1305, 1285, 1249, 1217 (C-OH), 1190, 1132, 1111 (C=S), 1083, 1056, 1032, 1017, 998, 981, 939, 872, 847, 803, 766, 748, 723; EI-MS (m/z, %): 310 (M^+, 100), 281 (4), 277 (5), 264 (5), 194 (22), 157 (19), 153 (45), 141 (10), 136 (5), 97 (4), 85 (4), 81 (3), 69 (9), 56 (9). Analysis: calcd. for $C_{14}H_{18}N_2O_4S$ (310.37): C 54.18, H 5.85, N 9.03%; found: C 54.21, H 5.89, N 9.07%.

(4-Benzylpiperazin-1-yl)(2,4-dihydroxyphenyl)methanethione (2)

STB (0.02 mol) and 1-benzylpiperazine (Alfa Aesar) (0.02 mol) were added to MeOH (50 mL) and heated to boiling (3 h). The mixture was hot filtered and water (100 mL) was added to the filtrate. The formed product was dissolved in a solution of Na_2CO_3 (5%) (50 mL) and filtered. The filtrate was then treated with a solution of HCl (10%) until acidic. The removed compound was washed by water and recrystallized from MeOH (50 mL).

Yield: 69%; m.p.: 78-80°C; log k = -0.073. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 9.69 (s, 1 H, HO-C2), 9.54 (s, 1 H, HO-C4), 8.58 (m, 1 H, H-C Ar), 7.78 (m, 1 H, H-C Ar), 7.38 (m, 2 H, H-C Ar), 6.97 (m, 2 H, H-C Ar), 6.26 (d, 1 H, *J* = 2.3 Hz, H-C3), 6.23 (dd, 1 H, *J* = 8.3 and 2.3 Hz, H-C5), 3.59 (s, 2 H, CH_2), 2.68 (m, 4 H, H-C piperazine), 2.33 (m, 4 H, H-C piperazine); IR (KBr, cm^{-1}): 3029 (OH, C Ar-H), 2916 (CH), 2809 (CH), 1617, 1594 (C=C), 1484, 1441 (C-H), 1293, 1213 (C-OH), 1113 (C=S), 1066, 1033, 1002, 978, 844, 798, 775, 746, 701; EI-MS (m/z, %): 328 (M^+, 32), 295 (12), 219 (4), 194 (8), 175 (6), 159 (32), 153 (16), 146 (36), 134 (21), 117 (5), 91 (100), 65 (5), 42 (17). Analysis: calcd. for $C_{18}H_{20}N_2O_2S$ (328.48): C 65.83, H 6.14, N 8.53%; found: C 65.98, H 6.16, N 8.57%.

(2,4-Dihydroxyphenyl)[4-(2-methoxyphenyl) piperazin-1-yl]methanethione (3)

STB (0.01 mol) and 1-(2-methoxyphenyl) piperazine (Alfa Aesar) (0.01 mol) were added to MeOH (50 mL) and heated to boiling (3 h). The mixture was hot filtered and the filtrate was left at room temperature (24 h). The filtrate was concentrated to dryness and recrystallized from aqueous (4 : 1) methanol (50 mL).

Yield: 63%; m.p.: 165-167°C; log k = -0.496. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 9.73 (s, 1 H, HO-C2), 9.50 (s, 1 H, HO-C4), 8.31 (s, 1 H, H-C Ar), 7.02-6.85 (m, 4 H, H-C Ar), 6.28 (d, 1 H, *J* = 2.2 Hz, H-C3), 6.23 (dd, 1 H, *J* = 8.4 and 2.2 Hz, H-C5), 3.78 (s, 3 H, CH_3), 3,60 (m, 4 H, H-C(piperazine), 3.2-3.1 (m, 4 H, H-C(piperazine); IR (KBr, cm^{-1}): 3246 (OH), 2955 (CH), 2833 (CH), 1617 (C=C), 1500 (C=C), 1439 (C-H), 1386, 1241 (C-OH), 1153, 1057, 1025, 976, 919, 845, 809, 751; EI-MS (m/z, %): 344 (M^+, 53), 328 (7), 311 (10), 298 (5), 258 (5), 224 (3), 200 (9), 191 (11), 175 (75), 162 (100), 153 (45), 150 (32), 147 (7), 131 (58), 120 (15), 117 (11), 106 (14), 97 (7), 91 (7), 81 (5), 77 (12), 69 (6), 65 (10), 56 (18), 51 (6), 45 (6), 41 (5), 39 (9). Analysis: calcd. for $C_{18}H_{20}N_2O_3S$ (344.43): C 62.77, H 5.85, N 8.13%; found: C 62.81, H 5.88, N 8.08%.

(2,4-Dihydroxyphenyl)[4-((2*E*)-phenylprop-2-ene-1-yl)piperazin-1-yl]methanethione (4)

STB (0.01 mol) and 1-[(2*E*)-3-phenylprop-2-en-1-yl]piperazine (Alfa Aesar) (0.01 mol) were added to MeOH (40 mL) and heated to boiling (3 h). The mixture was hot filtered and the removed compound was washed by water and recrystallized from aqueous (2 : 1) methanol (45 mL).

Yield: 69%; m.p.: 110-115°C; log k = -0.492. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 9.67 (s, 1 H, HO-C2), 9.51 (s, 1 H, HO-C4), 7.44 (d, 2 H, *J* = 8.7 Hz, H-C Ar), 7.33 (m, 2 H, H-C Ar), 7.23 (m, 1 H, H-C Ar), 6.98 (d, 1 H, *J* = 8.4 Hz, H-C Ar), 6.56 (d, 1 H, *J* = 16 Hz, H-C), 6.39 (m, 1 H, H-C), 6.26 (d, 1 H, *J* = 2.2 Hz, H-C3), 6.22 (dd, 1 H, *J* = 8.1 and 2.2 Hz, H-C5), 3.01 (m, 4 H, H-C piperazine), 3.18 (d, 2 H, *J* = 6.6 Hz, CH_2), 2.62 (m, 4 H, H-C piperazine); IR (KBr, cm^{-1}): 3024 (OH), 2921 (CH), 2818 (CH), 1614 (C=C), 1436, 1230 (C-OH), 1129 (C=S), 973, 843, 806, 785, 744; EI-MS (m/z, %): 354 (M^+, 10), 338 (3), 321 (7), 185 (17), 172 (21), 153 (56), 137 (9), 117 (100), 94 (5), 91 (13), 69 (7), 56 (14), 44 (15), 39 (5). Analysis: calcd. for $C_{20}H_{22}N_2O_2S$ (354.47): C 67.77, H 6.26, N 7.90; found: C 67.68, H 6.29, N 7.85%.

(2,4-Dihydroxyphenyl)(5-methoxy-1*H*-indol-1-yl)methanethione (5)

STB (0.0075 mol) and 2-methoxy-1*H*-indole (Alfa Aesar) (0.0075 mol) were added to MeOH (50 mL) and heated to boiling (3 h). The mixture was hot filtered and water (100 mL) was added to the filtrate. The removed compound was washed by water and recrystallized from aqueous (2 : 1) methanol (75 mL).

Yield: 66%; m.p.: 165-168°C; log k = -0.001. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 12.74 (s, 1 H, HO-C2), 10.39 (s, 1 H, HO-C4), 8.07 (d, 1 H, *J*

= 3.1 Hz, H-C indole 2), 7.86 (d, 1 H, *J* = 8.7 Hz, H-C indole 7), 7.71 (d, 1 H, *J* = 2.4 Hz, H-C indole 4), 7.45 (d, 1 H, *J* = 8.8 Hz, H-C6), 6.92 (dd, 1 H, *J* = 8.8 and 2.5 Hz, H-C indole 6), 6.68 (m, 1 H, H-C indole 3) 6.47 (dd, 1 H, *J* = 8.7 and 2.3 Hz, H-C5), 6.41 (d, 1 H, *J* = 2.3 Hz, H-C3), 3.83 (s, 3 H, CH_3); IR (KBr, cm^{-1}): 3312 (OH), 2942 (CH), 2833 (CH), 1624 (C=C), 1591 (C=C), 1559 (C=C), 1511 (C=C), 1471, 1436 (C-H), 1381, 1343, 1298, 1267, 1202 (C-OH), 1162, 1126 (C=S), 1071, 1022, 981, 922, 887, 836, 800, 784, 770, 746; EI-MS (m/z, %): 299 (M^+, 4), 297 (4), 283 (61), 266 (5), 174 (6), 147 (100), 142 (6), 137 (5), 132 (25). Analysis: calcd. for $C_{16}H_{13}NO_3S$ (299.34): C 64.20, H 4.38, N 4.68%; found: C 64.73, H 4.40, N 4.66%.

(2,4-Dihydroxyphenyl)(1*H*-imidazo[4,5-*b*]pyridin-1-yl)methanethione (6)

STB (0.01 mol) and 1*H*-imidazo[4,5-b]pyridine (Alfa Aesar) (0.01 mol) were added to MeOH (50 mL) and heated to boiling (3 h). The mixture was hot filtered and water (100 mL) was added to the filtrate. The removed compound was washed by water and recrystallized from aqueous (2:1) methanol (60 mL).

Yield: 61%; m.p.: 172-175°C; log k = -0.702. ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 11.89 (s, 1 H, HO-C2), 10.26 (s, 1 H, HO-C4), 8.49 (s, 1 H, H-C2'), 8.38 (m, 1 H, H-C5'), 8.05 (m, 1 H, H-C7'), 7.50 (d, 1 H, *J* = 8.8 Hz, H-C6), 6.69 (m, 1 H, H-C6'), 6.43 (d, 1 H, H-C3), 6.35 (m, 1 H, H-C5); IR (KBr, cm^{-1}): 3271 (OH), 3099 (C(Ar)-H), 2947 (CH), 1679 (C=N), 1616 (C=C), 1512 (C=C), 1459, 1391, 1334, 1262, 1164 (C-OH), 1129 (C=S), 1060, 1008, 971, 950, 886, 845, 810, 780; EI-MS (m/z, %): 270 (22), 184 (8), 167 (9), 153 (7), 135 (6), 119 (100), 92 (39), 77 (4), 65 (11), 57 (6), 41 (8), 38 (7). Analysis: calcd. for $C_{13}H_9N_3O_2S$ (271.29): C 57.55; H 3.34; N 15.49%; found: C 57.82; H 3.35; N 15.46%.

Antiproliferative assay *in vitro*

The following established *in vitro* human cell line was used in this study: HCV29T (bladder cancer) from the Fibiger Institute, Copenhagen, Denmark. Twenty-four hours before the addition of the tested agent, the cells were plated in 96-well plates (Sarstedt Inc.) at a density of 10^4 cells/well. Cell line was maintained in the opti-MEM medium supplement with 2 mM glutamine (Gibco), streptomycin (50 μg/mL), penicillin (50 U/mL) (Polfa, Tarchomin), and 5% fetal calf serum (Gibco). The cells were incubated at 37°C in a humid atmosphere saturated with 5% CO_2. The solutions of compounds (1 mg/mL) were prepared *ex tempore* by dissolving the substance in 100 μL of DMSO followed by addition of 900 μL of tissue culture medium. Afterwards, the compounds were diluted in the culture medium to final concentrations ranging from 0.1 to 100 μg/mL. The solvent (DMSO) used at the highest concentration in the test did not reveal any cytotoxic activity. Cisplatin was used as a test reference agent. The cytotoxicity assay was performed after 72 h exposure of the cultured cells at concentrations of tested agents ranging from 0.1 to 100 μg/mL. The SRB test was used to measure inhibition of cell proliferation *in vitro* (28). The cells attached to the plastic were fixed with cold 50% TCA (trichloroacetic acid, Sigma-Aldrich Chemie GmbH) added on the top of the culture medium in each well. The plates were incubated at 4°C for 1 h and then washed 5 times with tap water.

The background optical density was measured in the wells filled with the medium, without the cells. The cellular material fixed with TCA was stained with 0.4% sulforhodamine B (SRB, Sigma-Aldrich Chemie GmbH) dissolved in 1% acetic acid (POCh) for 30 min. The unbound dye was removed by rinsing (4 times) with 1% acetic acid, and the protein-bound dye was extracted with 10 mM unbuffered Tris base (tris(hydroxymethyl)aminomethane, POCh) for determination of optical density (at 540 nm) in a computer-interfaced, 96-well microtiter plate reader Uniskan II (Labsystems). The compounds were tested in triplicate for each experiment, and the experiments were repeated at least 3 times.

Antifungal activity of the compounds against fungi *Candida*

Fifty strains of *Candida albicans* taken from the mouth cavity of patients suffering from tumor diseases were used as the selective material. Itraconazole and fluconazole were administered for prophylaxis or due to the symptoms of candidosis. The isolates resistant to a number of drugs (5-fluorocytosine, ketoconazole, amphotericin B, itraconazole, miconazole, and fluconazole), as shown by Fungitest^R, were chosen for the dilution method testing of the compounds. The drug resistance was 56.7%; the resistance to itraconazole and fluconazole were 87.5% and 82.5%, respectively. In addition, the strain was used for comparison with the American Type Culture Collection (University Boulevard, Manassas, VA) *C. albicans* (ATCC 10231).

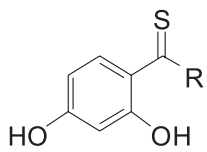
Six isolates of non-*albicans Candida* (two isolates of *C. tropicalis*, one of each of *C. glabrata, C.*

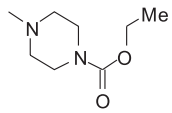
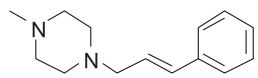
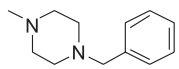
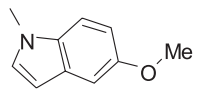
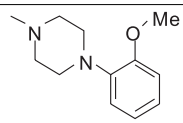
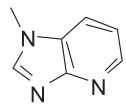
krusei, C. paratropicalis, and *C. tropicalis*) (n = 6) were tested. The drug resistance by Fungitest[R] was also determined for them. The yeast isolates were identified to the species level by the conventional morphological and biochemical methods by the Candi Select (Bio-Rad), Fungiscreen 4H (Bio-Rad), and Auxacolor (Bio-Rad) tests. The compounds were dissolved in 1% DMSO. The susceptibility testing was achieved by the agar dilution method. MIC values were determined by the agar dilution procedure according to the National Committee for Clinical Laboratory Standards (NCCLS) reference document M27 (29). The Sabouraud's medium (Bio-Rad) was used. Starting inocula were adjusted by the spectrophotometric densitometry (Bio-Merieux) to 1×10^{-5} CFU/mL. The concentrations of compounds were from 6.25 to 200 μg/mL. The plates were incubated at 37°C and read after 24 h of incubation. A solvent control was included in each set of assays; the DMSO solution at the maximum final concentration of 1% had no effect on the fungal growth. Itraconazole (Pliva, Kraków, Poland) and fluconazole (Janssen-Cilag) tested under the same experimental conditions were used as the references. MIC values expressed as the average value from 10 measurements for *C. albicans* and from 6 for non-*albicans Candida* (Table 2).

Activity of the compounds against phytopathogenic fungi

The test *in vitro* estimated inhibition of mycelium in the agar culture medium caused by the compound under investigation. The bioindicators: *Alternaria alternata, Botrytis cinerea, Rhizoctonia solani, Fusarium culmorum* and *Phytophthora cactorum* were used in the test. The solutions (suspensions) were prepared at the concentrations needed to obtain 200 and 20 μg/mL of the studied substance after dilution with the agar culture medium (PDA). Petri scale pans were used, into which the agar culture medium and the studied substance were poured. When the culture medium set, the infectious material of the tested fungus, in the form of agar disks overgrown with mycelium, was placed at three sites on its surface. After 3-5 days, depending on the mycelium culture, the linear growth of the mycelium colony was measured. The compound's action was determined as the percentage of mycelium growth inhibition compared with the control using the Abbott equation. Carbendazim and procymidon were used as the reference substances and tested under the same experimental conditions. The results are given in the four-degree scale, determined as the percentage of mycelium growth inhibition compared with the control (Table 3).

Table 1. Structure and antiproliferative activity of N,N-cyclic-2,4-dihydroxythiobenzamide derivatives against the cell of human HCV29T line expressed as IC_{50} (μg/mL).[a]



No.	R:	ID_{50} [μg/mL]	No.	R:	ID_{50} [μg/mL]
1		21.77 ± 6.00	**4**		24.28 ± 6.19
2		-[b]	**5**		10.51 ± 4.14
3		-[b]	**6**		33.98 ± 1.04
Cisplatin		0.93 ± 0.29			

[a]ID_{50}: indicates the compound concentration that inhibits the proliferation rate of tumor cells by 50% as compared to the control untreated cells. The values are the means ± SD of nine independent experiments. [b]Negative in the studied concentrations.

Table 2. Antifungal activity against *C. albicans* and *non-C. albicans* strains expressed in average MIC values [μg/mL].[a]

Compound	*C. albicans* ATCC 10231[b]	*C. albicans*[c] (n = 10)	*non-Candida albicans*[d] (n = 6)
1	170.0 ± 48.3	170.0 ± 48.3	-[e]
2	200.0 ± 0.0	190.0 ± 31.6	145.8 ± 84.3
3	200.0 ± 0.0	200.0 ± 0.0	166.7 ± 51.6
5	100.0 ± 0.0	60.0 ± 21.1	66.7 ± 37.6
6	200.0 ± 0.0	190.0 ± 31.6	-[e]
Itraconazole	200.0 ± 0.0	47.5 ± 7.9	27.5 ± 7.9
Fluconazole	200.0 ± 0.0	27.5 ± 7.9	12.5 ± 0.0

[a] MIC – the minimal inhibitory concentration caused full inhibition of growth in relation to the control [μg/mL]. [b] MIC ± SD (means ± standard deviation of three independent experiments). [c] MIC ± SD (MIC values expressed as the average value from 10 isolates ± standard deviation). [d] MIC ± SD (MIC values expressed as the average value from 6 isolates ± standard deviation). [e] Test was not performed.

Table 3. Antifungal activity of compounds against phytopathogenic fungi.[a]

Compound	Estimation of mycelium growth inhibition [mg/×L]									
	A. alternata		*B. cinerea*		*R. solani*		*F. culmorum*		*P. cactorum*	
	200	20	200	20	200	20	200	20	200	20
2	2	1	2	1	2	1	2	1	2	1
3	2	1	1	0	1	0	2	1	1	0
Carbendazim	-[b]	-[b]	-[b]	-[b]	3	3	3	3	3	3
Procymidon	2	2	3	3	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]

[a] The results are given in the four-degree scale determined in percent of mycelium growth inhibition compared with the control: 0 = 0-20%; 1 = 21-40%; 2 = 41-60%; 3 = 61-80%; 4 = 81-100%. [b] Test was not performed.

Biological studies were done in the Department of Pesticides Application, IPO, Warszawa, with the SPR/FA_2/11 procedures (certificate GLP Compliance No. G 013).

RESULTS AND DISCUSSION

Compounds **1**-**6** were formed by the reaction of the commercially available secondary cyclic amines with sulfinylbis[(2,4-dihydroxyphenyl) methanethione] (STB) in MeOH under reflux (3 h). The starting reagent STB was prepared from 2,4-dihydroxybenzenecarbodithioic acid and $SOCl_2$ in ethoxyethane (27). The yields of processes were in the range of 61-76%. Purity of the obtained compounds was checked by the reversed phase (RP-18) HPLC chromatography using the MeOH : H_2O mixture as a mobile phase. The structures of the obtained compounds are shown in Table 1. All these derivatives were characterized by spectral and elemental analysis data which confirmed their structures.

In the ^{1}H NMR spectra, the resonance signals of hydroxyl groups protons are usually registered as broad singlets in the range ca. 11.89 and 10.26 ppm. The protons of the piperazine are detected as two multiplies at 3.71 ppm and 3.15 ppm. The IR spectra show two strong bands in the region about 3312-3024 and 1241-1164 cm^{-1}, corresponding to ν (O-H) The signals of molecular ions M^+ (EI) are visible in the spectra of all compounds.

The antiproliferative activity of synthesized compounds has been evaluated against the cell of human HCV29T line (Table 1). Cisplatin was used as a reference drug. The cytotoxic activity *in vitro* was expressed as ID_{50} (μg/mL), the concentration of compound that inhibits proliferation rate of the tumor cells by 50%, as compared to the control untreated cells. The results of substance screening are summarized in Table 1. Antiproliferative activity *in vitro* of the presented compounds is varied - compound **5** proved to be the most active.

Antifungal activity of compounds was expressed as average MIC values against several clinical isolates of *C. albicans* (Table 2). Analyzing the results summarized in Table 2 it can be concluded that the activity of the compounds is varied and

depends significantly of the structure. The data of inhibitory effects indicate that, depending on the type of substitution, the obtained compounds are characterized by differentiated activities expressed in the MIC values ranging from 60 to 200 μg/mL. The strongest fungistatic activity is observed for studied compound **5** against *C. albicans* (MIC 60.0 μg/mL). Some derivatives were also tested for their activity against non-*albicans Candida*. The compounds are characterized by higher antifungal effects than those against the isolates of *C. albicans*. However, the compounds studied exhibit lower activity against *C. albicans* and non-*C. albicans* compared with standard substances. Our preliminary results may suggest some directions for further synthesis.

The results of *in vitro* screening against five strains of phytopathogenic fungi (*A. alternata, B. cinerea, R. solani, F. culmorum, and P. cactorum*) under the *in vivo* conditions are given in Table 3. Sarfun 500SC (carbendazim) and Sumilex 500SC (procymidone) were used as reference systems. In the laboratory studies at the concentration of 200 μg/mL, compound **2** revealed fungistatic action (at the level 41-60%) against all bioindicators. Compound **3** showed a particular inhibitory action, inhibiting growth of two pathogens – *A. alternata* and *F. culmorum*. This pathogens seem to be particularly susceptible to tested compounds.

CONCLUSION

Novel N,N-cyclic-2,4-dihydroxythiobenzamide derivatives were formed by the reaction of the commercially available reagents with STB. The compounds show antiproliferative activity *in vitro* against the HCV29T cancer cell lines. They are also characterized by antifungal properties against *Candida, non-Candida* and phytopathogenic fungi. Following on from these results, one may synthesize a larger series of thiobenzamide derivatives in order to further investigate these findings.

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SYNTHESIS AND *IN VITRO* ANTIPROLIFERATIVE ACTIVITY OF NOVEL 2-ARYLIDENEAMINOBENZIMIDAZOLE DERIVATIVES

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Abstract A new class of Mannich bases **9-26**, derivatives of 2-amino-1H-benzimidazole, were obtained in the condensation of Schiff bases **1-4** or 2-benzylaminobenzimidazoles **5-8** with selected secondary amines: morpholine, piperidine, N-methylpiperazine, N-phenylpiperazine, 1-(2-pyridyl)piperazine, 1(2-methoxyphenyl)piperazine, 1-(2-pyrimidinyl)piperazine and formaldehyde in ethanol. The pyrimido[1,2-a]benzimidazole derivatives **27-29** have been synthesized in the reactions of Schiff base **2** with selected compounds containing active methylene group: acetylacetone, benzoylacetone and malononitrile. The structures **1-29** were confirmed by the results of elementary analysis and their IR, ^{1}H- and ^{13}C-NMR spectra. The products **1-29** are of interest for biological studies and can be substrates for further synthesis. All compounds were screened against the cells of MV4-11 human leukemia and then the most active of them **5**, **7**, **9-16**, **24-26**, **28**, **29** were tested towards human T47D breast and A549 lung cancer cells as well as normal mouse fibroblasts (BALB/3T3). The most active compound against the cancer cell lines was 4-amino-3-cyano-2-(4-hydroxyphenylene)-1,2-dihydropyrimido[1,2-a]benzimidazole (**29**) (IC_{50} 0.23 ± 0.05 μg/mL against MV4-11 cells) showing in parallel very low cytotoxicity towards mouse fibroblasts. Cisplatin was the control drug.

Keywords: 2-arylideneaminobenzimidazole, 2-benzylaminobenzimidazole, Mannich bases, pyrimido[1,2-a]benzimidazole, antiproliferative activity *in vitro*

The Mannich reaction is very important for the synthesis of biologically active compounds (1). Many studies have shown that Mannich bases possess potent biological activities: analgesic (2), antimalarial (3), anticonvulsant (4, 5), antipsychotic (6) or antimicrobial (7-10). The structure of the Mannich bases possess currently used drugs (11) with diverse pharmacological activity e.g., tramadol - an opioid pain reliever, procyclidine - used for the treatment of drug-induced parkinsonism, molindon - neuroleptic, falicain - used for local anesthesia in laryngology and rolitetracycline - a tetracycline antibiotic (Fig. 1).

The problem of treatment of neoplastic diseases forces to search for new compounds, also Mannich bases. The most recent medicinal chemistry publications (12-23) have reported that the Mannich bases, derivatives of the various heterocycles, show in preliminary tests antiproliferative activity *in vitro* against human tumor cell lines. In a few publications (12, 13), the authors revealed the possible mechanism of their antitumor effects by molecular docking studies.

In our recently published work (14) Mannich bases, derivatives of 2-thioxo-imidazo[4,5-*b*]pyridine, exhibited good antiproliferative activity *in vitro* against cancer cell lines: breast (MCF-7), lung (A549) and leukemia (MV4-11). The most active and in parallel selective towards cancer cells was 1-benzyl-6-bromo-3-morpholinemethyl-2-thioxoimidazo[4,5-b]pyridine. Compounds derived from 4-piperazinylquinoline and isatin (15) exhibited promising anticancer activity *in vitro* against human breast cancer cell lines (MDA-MB468, MCF-7). The Mannich bases, derivatives of 3-aminomethyl-2-aryloimidazo[2,1-b]benzothiazole were active against two breast cancer cell lines (MCF-7, HeLa) and liver cancer cell line (HepG2). The most active, among all obtained compounds, was 3-pyrrolidineamino-2-phenyl-6-fluoroimidazo[2,1-b]benzotiazole (16).

There is a new approach in the drug discovery that combines two or more pharmacophores with

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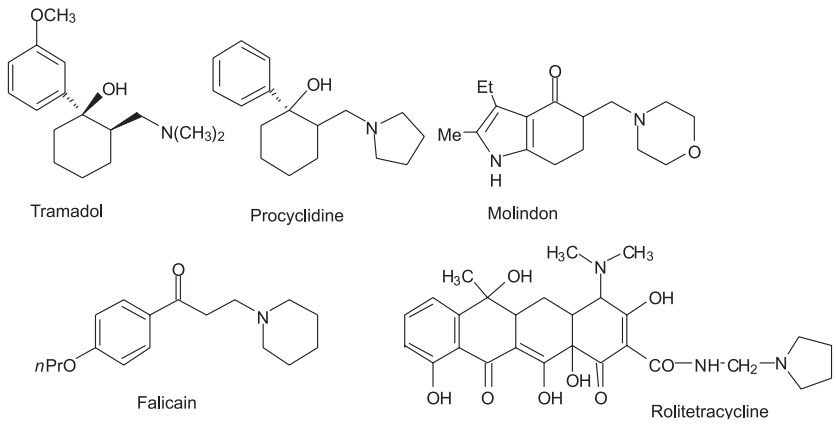


Figure 1. Selected drugs – Mannich bases

anticancer activity into a single molecule. Schiff and Schiff-Mannich bases are also common pharmacophores in the design and development of anticancer agents. Schiff bases also demonstrate interesting pharmacological activities including antidiabetic (24), antioxidant (25), antimicrobial (26-28), antitumor and anticancer (29) activities.

Significant activity against breast cancer line (MCF-7) exhibited Mannich-Schiff base derivatives of isatin-benzmidazole and isatin-thiazoline (17). Higher activities possess isatin-benzimidazole derivatives. They contained in their structures substituents e.g., morpholinmethyl, piperidinmethyl, N-phenylpiperazinemethyl, N-(2-methoxyphenyl)piperazinemethyl or N-methylpiperazinemethyl. The cytotoxic effects *in vitro* against liver cancer cell line (HepG2) demonstrated Schiff and Mannich bases, derivatives of 5-substituted-4-amino-3-thioxo-1,2,4-triazole (18).

Another way to search for biologically active compounds, including the anticancer activity, is to modify the chemical structure of known drugs of various pharmacological activity or natural compounds. This chemical modification could change the profile of action of new synthesized compounds. An interesting example was the use of ofloxacin – antibacterial drug, as a substrate in Schiff and then Mannich condensations with N-methylpiperazine and formaldehyde. New products which possess anticancer activity *in vitro*, have been obtained (19). *Via* the Mannich condensation, anticancer prodrugs of anthracycline antibiotics: doxorubicin and daunorubicin, which contain in their structure polyethylene glycols (PEGs), have been synthesized. PEGs are potential drug carriers for improving the therapeutic index of anticancer agents. Obtained prodrugs, at the same dose, showed comparable cytotoxicity as anthracyclines, but have prolonged time of action (20). 6α7β-Dihydroxyvoucapan-17β-oic acid (21), isolated from the fruit of the Brazilian plant *Pterodon polygalaeflorus*, possesses anti-inflammatory and analgesic activity. The aminomethylation of this diterpenoid led to six compounds, which showed antiproliferative activity *in vitro* against nine human cell lines (UACC-62, MCF-7, NCI-ADR/RES, 786-0, NCI-H460, PC-3, OVCAR-036, HT-29, K562).

Synthesis of new 2-aminobenzimidazole derivatives, possessing anticancer activity, is now one of the most important directions of research conducted on this group of compounds. 2-Aminobenzimidazole-based compounds demonstrated high cytotoxic activity against human cancer cell lines: lung (A549), breast (MCF-7) and leukemia (HL-60) (30). The main goal of this paper was to synthesise Schiff bases, in reaction of 2-aminobenzimidazole with selected aromatic aldehydes and its chemical modification: by reduction of obtained imines, Mannich condensation or/and reaction with selected compounds containing active methylene group. All the synthesised compounds were examined for their antiproliferative activity *in vitro* against human cancer cell lines: leukemia (MV4-11), breast (T47D) and lung (A549) and also mouse fibroblast cell line (BALB/3T3).

EXPERIMENTAL

Chemistry

Melting points were measured with a Boetius melting point apparatus. The new products were analyzed using a Perkin Elmer 2400 analyzer. IR spectra (in KBr) were recorded with an IR 75 spec-

trophotometer, ^{1}H- and ^{13}C NMR spectra – on a Bruker AVANCE DRX 300 MHz apparatus using DMSO-d_6 as an internal standard. The course of reaction and the purity of products were checked by TLC (Kieselgel G, Merck) in diethyl ether : ethanol 5 : 1, v/v as eluent.

The synthesis of 2-aminobenzimidazole (31) and Schiff base **4** (32) have been presented in our previous articles.

General procedure for the preparation of compounds 1-4

Reaction of 2-aminobenzimidazole with selected aromatic aldehydes: salicylic, 4-hydroxy-, 3-hydroxy- and 2-chlorobenzaldehyde. To a solution of 2-aminobenzimidazole (0.01 mol) in ethanol (30 mL) appropriate aromatic aldehydes were added and catalyst Triflate was used. The solution was refluxed ca. 8-10 h (TLC control). After cooling, the precipitate was filtered, washed with diethyl ether, dried and crystallized from appropriate solvent.

2–(Salicylideneamino)benzimidazole (1)

Obtained as a yellow precipitate, yield 1.80 g (76%); crystallized from ethanol; m.p. 222-223°C; IR (KBr, cm^{-1}): 3650 (OH), 3420 (NH), 3080, 1600 (ring), 1520 (C=C, C=N), 1260, 1230 (OH), 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 7.01 (m, 2H, Ar-H), 7.20 (m, 2H, Ar-H), 7.48 (m, 3H, Ar-H), 7.86 (d, 1H, *J* = 7.2 Hz, Ar-H), 9.67 (s, 1H, -CH=N-), 12.13 (s, 1H, -OH), 12.73 (s, 1H, -NH, imidazole); Analysis: calcd. for $C_{14}H_{11}N_3O$ (237.26): C 70.87, H 4.67, N 17.71%; found: C 70.47, H 4.63, N 17.70%.

2-(4-Hydroxybenzylideneamino)benzimidazole (2)

Obtained as a yellow precipitate, yield 1.88 g (79%); crystallized from ethanol; m.p. 267-269°C; IR (KBr, cm^{-1}): 3360 (NH), 3080 (CH arom.), 1635 (CH=N, ring), 1500, 1520 (C=C), 1260 (C-O-H), 840 (CH), 730 (CH); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 6.95 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.15 (m, 2H, Ar-H), 7.47 (m, 2H, Ar-H), 7.92 (d, 2H, *J* = 8.7 Hz, Ar-H), 9.31 (s, 1H, -CH=N-), 10.44 (s, 1H, -OH), 12.51 (s, 1H, -NH-, imidazole). Analysis: calcd. for $C_{14}H_{11}N_3O$ (237.26): C 70.87, H 4.67, N 17.71%; found: C 70.53, H 4.84, N 17.16%.

2-(3-Hydroxybenzylideneamino)benzimidazole (3)

Obtained as a yellow precipitate, yield 1.82 g (77%); crystallized from toluene; m.p. 221-223°C; IR (KBr, cm^{-1}): 3380 (NH), 3070 (-CH-), 1680 (CH=N ring), 1230 (C-O-H), 890, 780 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 7.04 (m, 1H, Ar-H); 7.18 (m, 2H, Ar-H); 7.38 and 7.49 (t, 5H, *J* = 7.8 Hz + m, Ar-H); 9.37 (s, 1H, -CH=N-); 9.87 (s, 1H, -OH); 12.67 (s, 1H, -NH-, imidazole). Analysis: calcd. for $C_{14}H_{11}N_3O$ (237.26): C 70.87, H 4.67, N 17.71%; found: C 70.82, H 4.90, N 17.59%.

General procedure for the preparation of compounds 5-8

Reduction of azamethine bond in Schiff bases. To a solution of Schiff bases **1-5** (0.01 mol) in isopropanol (60 mL) $NaBH_4$ (0.01 mol) was added. The solution was refluxed for ca. 6 h. The solvent was evaporated under reduced pressure, 200 mL of cold water and ice was added to cold and dry residue. The precipitate formed was filtered, washed with water to neutral reaction. After drying, precipitates were crystallized from appropriate solvent.

2-(Salicylamino)benzimidazole (5)

Obtained as a white precipitate, yield 1.82 g (76%); crystallized from ethanol; m.p. 225-226°C; IR (KBr, cm^{-1}): 3420 (NH), 2920 (-CH_2-), 1600 (-CH-, arom.), 1280 (C-OH), 760 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 4.39 (d, 2H, *J* = 5.30 Hz, -*CH_2*-NH-), 6.83 (m, 2H, Ar-H), 6.92 (m, 2H, Ar-H), 7.17 (m, 4H, Ar-H), 7.30 (br, 1H, -CH_2-*NH*-), 9.70 (s, 1H, -OH), 11.67 (s, 1H, -NH-, imidazole). Analysis: calcd. for $C_{14}H_{13}N_3O$ (239.27): C 70.28, H 5.48, N 17.56%; found: C 70.17, H 5.47, N 17.53%.

2-(4-Hydroxybenzylamino)benzimidazole (6)

Obtained as a white precipitate, yield 1.82 g (76%); crystallized from ethanol; m.p. 225-226°C; IR (KBr, cm^{-1}): 3420 (NH), 3200 (OH), 2920 (CH), 2850 (-CH_2-), 1600 (C=C), 1280 (C-OH), 1175, 820, 760; ^{1}H NMR (300 MHz, DMSO, δ, ppm): 4.45 (d, 2H, *J* = 5.4Hz, -*CH_2*-NH-), 7.10 (br, 1H, -CH_2-*NH*-), 7.12 (m, 2H, Ar-H), 7.35 (d, 2H, *J* = 8.6 Hz, Ar-H), 7.60 (m, 2H, Ar-H), 8.03 (d, 2H, *J* = 8.6 Hz, Ar-H), 9.30 (s, 1H, -OH); 11.0 (s, 1H, -NH-, imidazole). Analysis: calcd. for $C_{14}H_{13}N_3O$ (239.27): C 70.28, H 5.48, N 17.56%; found: C 70.16, H 5.75, N 17.23%.

2-(3-Hydroxybenzylamino)-1H-benzimidazole (7)

Obtained as a white precipitate, yield 1.76 g (74%); crystallized from ethanol; m.p. 200-202°C; IR (KBr, cm^{-1}): 3410 (NH), 3080 (CH arom.), 2920 (-CH_2-), 1280 (-C-O-H), 880, 730 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 4.43 (d, 2H, *J* = 5.5 Hz, -*CH_2*-NH-); 6.61 + 6.80 +7.08 (m, 9H, -CH_2-*NH*- and Ar-H); 9.39 (s, 1H, -OH), 11.76 (s, 1H, -

NH-, imidazole). Analysis: calcd. for $C_{14}H_{10}ClN_3$ (255.7): C 70.28; H 5.48; N 17.56%; found: C 70.44; H 5.62; N 17.55%.

2-(2-Chlorobenzylamino)-1H-benzimidazole (8)

Obtained as a white precipitate, yield 2.42 g (94%); crystallized from dioxane; m.p. 104-106°C; IR (KBr, cm^{-1}): 3080 (CH arom.); 3300 (NH); 2845 (-CH_2-); 1600 (NH); 1575 (ring); 770 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 4.59 (d, 2H, *J* = 5,4 Hz, -*CH*$_2$-NH-); 6.87 (m, 2H, Ar-H); 7.12 (m, 3H, -CH_2-*NH*- and Ar-H); 7.27 (m, 2H, Ar-H); 7.44 (m, 2H, Ar-H); 10.87 (s, 1H, -NH-, imidazole). Analysis: calcd. for $C_{14}H_{13}N_3O$ (239.5): C 65.25, H 4.88, N 16.30%; found: C 65.06, H 5.30, N 15.96 %.

General procedure for the preparation of 9-26

The Mannich reaction. To a solution of appropriate Schiff bases **1-4** (0.01 mol) or 2-arylaminobenzimidazoles **5-8** (0.01 mol) in ethanol (25 mL) selected secondary amines: morpholine, piperidine, N-methylpiperazine, N-phenylpiperazine, 1-(2-pyridyl)piperazine, 1(2-methoxyphenyl)piperazine, 1-(2-pyrimidinyl)piperazine (0.01 mol) and 37% formaldehyde (0.03 mol) were added. The mixture was stirred at room temperature for ca. 4-6 h (TLC control). The precipitate formed was filtered, washed with diethyl ether, dried and crystallized form appropriate solvent.

1-[(Piperidin-1-yl)methyl]-2-(salicylideneamino) benzimidazole (9)

Obtained as a yellow precipitate, yield 2.10 g (63%); crystallized from ethanol; m.p. 149-151°C; IR (KBr, cm^{-1}): 3030 (CH, arom.), 2920 (-CH_2-), 1600 (ring), 1580 (C=N), 1180 (C-O-H), 760 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.45 (m, 6H, -CH_2-CH_2-CH_2-, piperidine), 2.49 (m, 4H, -CH_2-N-CH_2-, piperidine), 5.11 (s, 2H, -CH_2-), 6.99 (m, 2H, -Ar-H), 7.23 (m, 2H, Ar-H), 7.49 (m, 1H, Ar-H), 7.64 (m, 2H, Ar-H), 7.97 (d, 1H, *J* = 6.6 Hz, Ar-H), 9.65 (s, 1H, -CH=N-), 9.72 (s, 1H, -OH). ^{13}C NMR (300 MHz, DMSO, δ, ppm): 165.57, 160.35, 154.31, 140.99, 135.90, 134.91, 131.28, 122.33, 122.24, 120.03, 119.75, 118.59, 116.82, 111.32, 64.46, 51.25 (2C), 25.33 (2C), 23.39; Analysis: calcd. for $C_{20}H_{22}N_4O$ (334.41): C 71.83, H 6.63, N 16.75%; found: C 71.64, H 6.54, N 16.40%.

1-[(Morpholin-4-yl)methyl]-2-(salicylideneamino)benzimidazole (10)

Obtained as a yellow precipitate, yield 2.28 g (68%); crystallized from ethanol; m.p. 153-154°C; IR (KBr, cm^{-1}): 2920 (-CH_2-), 1600 (ring), 1580 (C=N), 1180 (C-O-H), 760 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.56 (m, 4H, -CH_2-N-CH_2-, morpholine), 3.53 (m, 4H, -CH_2-O-CH_2-, morpholine), 5.13 (s, 2H, -CH_2-), 7.02 (m, 2H, Ar-H), 7.24 (m, 2H, Ar-H), 7.49 (m, 1H, Ar-H), 7.65 (m, 2H, Ar-H), 7.98 (m, 1H, Ar-H), 9.65 (s, 1H, CH=N-), 9.74 (s, 1H, -OH). ^{13}C NMR (300 MHz, DMSO, δ, ppm): 164.51, 160.38, 154.29, 141.03, 135.75, 134.92, 131.21, 122.43, 122.32, 120.08, 119.76, 118.67, 116.80, 111.17, 66.06 (2C), 63.73, 50.46 (2C); Analysis: calcd. for $C_{14}H_{13}N_3O$ (239.5): C 67.84, H 5.99, N 16.66%; found: C 67.61, H 5.67, N 16.54%.

1-[(4-Methyl-piperazin-1-yl)methyl]-2-(salicylideneamino)benzimidazole (11)

Obtained as a yellow solid, yield 1.88 g (51%); crystallized from DMF; m.p. 157-161°C; IR (KBr, cm^{-1}): 2950 (CH_3), 2920 (-CH_2-), 1600 (ring), 1540 (C=N), 1180 (C-O-H), 760 (CH, arom.). ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.13 (s, 3H, -CH_3), 2.42 (m, 4H, -CH_2-N-CH_2-, piperazine), 2.80 (m, 4H, -CH_2-N-CH_2-, piperazine), 5.13 (s, 2H, -CH_2-), 7.03 (d, 2H, *J* = 7.8 Hz, Ar-H), 7.24 (m, 2H, Ar-H), 7.58 (m, 3H, Ar-H), 7.97 (d, 1H, *J* = 7.2 Hz, Ar-H), 9.41 (s, 1H, -CH=N-), 9.73 (s, 1H, -OH). ^{13}C NMR (300 MHz, DMSO, δ, ppm): 164.65, 160.33, 154.32, 141.00, 135.90, 134.90, 131.27, 122.32, 122.23, 120.03, 118.60, 116.81, 116.73, 111.33, 64.46, 55.34 (2C), 51.25 (2C), 43.39; Analysis: calcd. for $C_{20}H_{23}N_5O$ (349.43): C 68.74, H 6.63, N 20.04%; found: C 68.02, H 6.72, N 20.01%.

1-(Piperidin-1-yl)-2-(4-hydroxybenzylideneamino)benzimidazole (12)

Obtained as a yellow precipitate, yield 0.841 g (25%); crystallized from ethanol; m.p. 187-190°C; IR (KBr, cm^{-1}): 2940 (-CH_2-), 1600 (ring), 1450 (C=N), 1140 (C-O-H), 840 (CH), 760 (CH); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.49 (m, 6H, -CH_2-CH_2-CH_2-, piperidine), 2.55 (m, 4H, -CH_2-N-CH_2-, piperidine), 5.19 (s, 2H, -CH_2-), 6.96 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.18 (m, 2H, Ar-H), 7.60 (m, 2H, Ar-H), 7.92 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.97 (d, 1H, *J* = 8.4Hz, Ar-H), 9.34 (s, 1H, -CH=N-), 9.72 (s, 1H, -OH). ^{13}C NMR (300 MHz, DMSO, δ, ppm): 158.24, 154.64, 152.82, 141,50, 137.23, 136.58, 128.27, 126.48, 121.63 (2C), 118.25, 115.97 (2C), 111.19, 64.08), 51.75 (2C), 25.46 (2C), 23.41; Analysis: calcd. for $C_{20}H_{22}N_4O_1$ (334.41): C 70.94, H 5.54, N 17.03%; found: C 71.02, H 5.62, N 16.93%.

1-(Morpholin-4-yl-methyl)-2(-4-hydroxybenzylideneamino)benzimidazole (13)

Obtained as a yellow precipitate, yield 1.59 g (47%); crystallized from dioxane; m.p. 200-204°C; IR (KBr, cm^{-1}): 2940 (-CH_2-), 1600 (ring), 1450 (C=N ring), 1360 (NH), 1140 (C-O-H), 840, 760 (CH); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.58 (m, 4H, CH_2-N-CH_2-, morpholine), 3.55 (m, 4H, -CH_2-O-CH_2-, morpholine), 5.21 (s, 2H, -CH_2-), 6.95 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.23 (m, 2H, Ar-H), 7.61 (m, 2H, Ar-H), 7.98 (d, 2H, *J* = 8.4 Hz, Ar-H), 9.36 (s, 1H, -CH=N-), 9.46 (s, 1H, -OH); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 164.59, 160.27, 155.79, 141.21, 138.54, 136.77, 131.97 (2C), 122.11, 121.77, 118.34, 116.62, 116.06, 111.08, 66.17 (2C), 63.32, 50.42 (2C). Analysis: calcd. for $C_{19}H_{20}N_4O_2$ (336.39): C 70.43, H 5.62, N 16.97%; found: C 70.71, H 5.23, N 16.63%.

1-[(Piperidin-1-yl)methylo]-2-(3-hydroxybenzylideneamino)benzimidazole (14)

Obtained as a yellow precipitate, yield 1.60 g (48%), crystallized from toluene; m.p. 161-163°C; IR (KBr, cm^{-1}): 3080 (CH arom.), 2940 (-CH_2-), 1610 (arom., ring), 1480 (C=N ring), 1280 (C-O-H), 900, 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.24 (m, 2H, -CH_2-*CH_2*-CH_2-, piperidine); 1.46 (m, 4H, -*CH_2*-CH_2-*CH_2*-, piperidine); 2.56 (m, 4H, -*CH_2*-*N*-*CH_2*-, piperidine); 5.22 (s, 2H, Ar-H); 7.04 (m, 1H, Ar-H); 7.22 (m, 1H, Ar-H); 7.39 (t, 2H, *J* = 7.8 Hz, Ar-H); 7.60 (m, 4H, Ar-H); 9.40 (s, 1H, -OH); 9.86 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.25, 157.8, 155.15, 141.14, 136.99, 130.15, 128.84), 127.58, 122.21, 122.08, 120.32, 118.60, 114.77, 111.43, 64.28, 51.20 (2C), 25.47 (2C), 23.39. Analysis: calcd. for $C_{20}H_{22}N_4O$ (334.4): C 71.83, H 6.63, N 16.75%; found: C 72.23, H 6.77, N 16.79%.

1-[(Morpholin-1-yl)methyl]-2-(3-hydroxybenzylideneamino)benzimidazole (15)

Obtained as a yellow precipitate, yield 2.05 g (61%); crystallized from toluene; m.p. 163-165°C; IR (KBr, cm^{-1}): 3080 (CH), 2920 (-CH_2-), 1610 (arom., ring), 1480 (C=N ring), 1280 (C-O-H), 900, 780 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.59 (t, 4H, *J* = 4.2 Hz, -CH_2-N-CH_2-, morpholine); 3.54 (t, 4H, *J* = 4.2 Hz, -CH_2-O-CH_2-, morpholine); 5.23 (s, 2H, -CH_2-); 7.04 (m, 1H, Ar-H); 7.24 (m, 2H, Ar-H); 7.40 (m, 1H, Ar-H); 7.54 (m, 2H, Ar-H); 7.62 (m, 1H, Ar-H); 7.95 (m, 1H, Ar-H); 9.41 (s, 1H, -OH); 9.84 (s, 1H, -CH=N-). ^{13}C NMR (300 MHz, DMSO, δ, ppm): 159.85, 158.78, 155.07, 141.11, 136.50, 135.93, 130.15, 122.37, 122.21, 121.65, 120.37, 118.68, 114.93, 111.31, 66.02 (2C), 63.47, 50.41 (2C); Analysis: calcd. for $C_{19}H_{20}N_4O$ (336,4): C 67.84, H 5.99, N 16.66%; found: C 69.45, H 6.00, N 16.20%.

1-[(4-Phenylpiperazin-1-yl)methyl]-2-(3-hydroxybenzylideneamino)benzimidazole (16)

Obtained as a yellow precipitate, yield 2.84 g (64%); crystallized from toluene; m.p. 149-150°C; IR (KBr, cm^{-1}): 3010 (CH arom.), 2940 (-CH_2-), 1610 (arom., ring), 1480 (C=N ring), 1280 (C-O-H), 800, 770 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.74 (t, 4H, -CH_2-N-CH_2-, piperazine); 3.09 (t, 4H, -CH_2-N-CH_2-, piperazine); 5.31 (s, 2H, -CH_2-); 6.70 (t, 1H, *J* = 7.2 Hz, Ar-H); 6.84 (d, 1H, *J* = 7.8 Hz, Ar-H); 7.13 (m, 6H, Ar-H); 7.36 (t, 1H, *J* = 7.8 Hz, Ar-H); 7.56 (m, 2H, Ar-H); 7.73 (m, 2H, Ar-H), 9.41 (s, 1H, -OH); 9.61 (s, 1H, -CH=N-). Analysis: calcd. for $C_{25}H_{25}N_5O$ (411.50): C 72.97, H 6.12, N 17.02%; found: C 72.82, H 6.28, N 17.27%.

1-[((4-Pyridin-2-yl)piperazin-1-yl)methyl]-2-(3-hydroxybenzylideneamino)benzimidazole (17)

Obtained as a yellow precipitate, yield 1.45 g (35%); crystallized from toluene; m.p. 199-200°C; IR (KBr, cm^{-1}): 3010 (CH arom.), 2920 (-CH_2-), 1610 (arom.), 1480 (C=N ring), 1260 (C-O-H), 890, 780 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.49 (m,4H, -CH_2-N-CH_2-, piperazine); 3.26 (m, 4H, -CH_2-N-CH_2-, piperazine); 5.29 (s, 2H, -CH_2-); 6.61 (m, 1H, Ar-H); 7.38 (m, 8H, Ar-H); 8.28 (m, 3H, Ar-H); 9.38 (s, 1H, -OH); 9.85 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.84, 157.83, 155.43, 155.09, 149.51, 141.10, 136.51, 135.81, 135.17, 127.5, 122.40, 122.21, 121.66, 121.32, 120.39, 119.9, 118.68, 114.85, 109.99, 63.42, 49.85 (2C), 43.18 (2C); Analysis: calcd. for $C_{24}H_{22}N_6O$ (412,49): C 69.88, H 5.86, N 20.37%; found: C 69.64, H 5.62, N 20.57%.

1-[(4-(2-Methoxyphenyl)piperazin-1-yl)methyl]-2-(3-hydroxybenzylideneamino)-benzimidazole (18)

Obtained as a yellow precipitate, yield 2.39 g (54%); crystallized from toluene; m.p. 147-149°C; IR (KBr, cm^{-1}): 3040 (CH arom.), 2940 (-CH_2-), 2840 (Ar-OCH_3), 1610 (arom., ring), 1480 (C=N), 1280 (C-O-H), 910, 770 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.74 (m, 4H, -CH_2-N-CH_2-, piperazine), 2.98 (m, 4H, -CH_2-N-CH_2-, piperazine), 3.65 (s, 3H, -OCH_3); 5.31 (s, 2H, -CH_2-); 6.84 (m, 4H, Ar-H); 7.04 (m, 1H, Ar-H); 7.24 (m, 2H, Ar-H); 7.39 (m, 1H, Ar-H); 7.52 (m, 3H, Ar-H); 7.71 (m, 1H, Ar-H); 9.41 (s, 1H, -OH); 9.85 (s, 1H, -CH=N-). Analysis: calcd. for $C_{26}H_{27}N_5O_2$ (441.53): C 70.73, H 6.16, N 15.86%; found: C 70.65, H 6.19, N 15.83%.

1-[(Piperidin-1-yl)methyl]-2-(2-chlorobenzylideneamino)benzimidazole (19)

Obtained as a yellow precipitate, yield 2.65 g (75%); crystallized from toluene; m.p. 123-125°C; IR (KBr, cm^{-1}): 3050 (CH) 2940, 2870 (-CH_2-); 1600 (ring); 1480 (C=N); 750 (-CH_2-); 770 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.45 (m, 6H, -CH_2-CH_2-CH_2-, piperidine); 2.45 (m, 4H, -CH_2-N-CH_2-, piperidine); 5.26 (s, 2H, -CH_2-); 7.23 (m, 2H, Ar-H); 7.59 (m, 1H, Ar-H); 7.68 (m, 4H, Ar-H); 8.74 (d, 1H, *J* = 7.5 Hz, Ar-H); 9.81 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.29, 154.62, 141.04, 136.21, 136.09, 134.26, 131.87, 130.39, 128.72, 127.98, 127.86, 122.48, 118.95, 111.64, 64.31, 51.13 (2C), 25.45 (2C), 23.36; Analysis: calcd. for $C_{20}H_{21}N_4Cl$ (352.9): C 68.08, H 6.00, N 15.88%; found: C 68.44, H 6.07, N 15.91%.

1-[(4-Phenylpiperazin-1-yl)methyl]-2-(2-chlorobenzylideneamino)-1H-benzimidazole (20)

Obtained as a yellow precipitate, yield 2.45 g (56%); crystallized from toluene; m.p. 174-175°C; IR (KBr, cm^{-1}): 3075 (CH arom.); 2925 (-CH_2-); 1590 (ring); 1500 (C=N); 750 (CH arom.). ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.75 (m, 4H, CH_2-N-CH_2-, piperazine); 3.10 (m, 4H, -CH_2-N-CH_2-, piperazine); 5.38 (s, 2H, -*CH_2*-); 6.72 (d, 1H, *J* = 7.24 Hz, Ar-H); 6.85 (d, 2H, *J* = 8.1 Hz, Ar-H); 7.14 and 7.27 (m, 4H, Ar-H); 7.63 (m, 4H, Ar-H); 8.32 (d, 1H, *J* = 7.5 Hz, Ar-H); 9.84 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.55, 154.69, 150.84, 141.02, 136.27, 135.90, 134.39, 131.85, 130.42, 128.96, 128.94 (2C), 128.75, 128.05, 122.64, 119.03, 118.84, 115.53 (2C), 111.62, 63.29, 49.87 (2C), 48.42 (2C); Analysis: calcd. for $C_{25}H_{24}N_5Cl$ (429.9): C 69.84, H 5.63, N 16.29%; found: C 70.15, H 5.78, N 16.22%.

1-[(4-(Pyridin-2-yl)piperazin-1-yl)methyl]-2-(2-chlorobenzylideneamino)-1H-benzimidazole (21)

Obtained as a yellow precipitate, yield 2.48 g (57%); crystallized from toluene; m.p. 168-169°C; IR (KBr, cm^{-1}): 3010 (CH); 2950 (-CH_2-); 1600 (ring); 1480 (C=N); 775, 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.78 (t, 4H, -CH_2-N-CH_2-, piperazine); 3.54 (t, 4H, CH_2-N-CH_2-, piperazine); 5.28 (s, 2H, -CH_2-); 6.56 (m, 2H, Ar-H); 7.28 (m, 2H, Ar-H); 7.40 (m, 2H, Ar-H); 7.50 (m, 3H, Ar-H); 7.77 (m, 1H, Ar-H); 8.18 (m, 1H, Ar-H); 8.40 (d, 1H, *J* = 7.5 Hz, Ar-H); 9.94 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 161.62, 159.14, 154.89, 149.15, 141.73, 137.60, 137.41, 135.94, 135.47, 132.72, 130.45, 128.79, 127.14, 122.99, 122.88, 119.68, 113.28, 110.67, 106.98, 64.22, 50.46 (2C), 45.07 (2C); Analysis: calcd. for $C_{24}H_{23}N_6Cl$ (430.9): C 66.89, H 5.38, N 19.50%; found: C 67.02, H 5.44, N 19.80%.

1-[(4-(2-Methoxyphenylene)piperazin-1-yl)methyl]-2-(2-chlorobenzylideneamino)-1H-benzimidazole (22)

Obtained as a yellow precipitate, yield 3.22 g (69%); crystallized from toluene; m.p. 159-161°C; IR (KBr, cm^{-1}): 3080 (CH arom.); 2940 (-CH_2-); 2850 (-OCH_3); 1600 (ring); 1450 (-CH_2-); 750 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.35 (m, 4H, -CH_2-N-CH_2-, piperazine); 2.91 (m, 4H, -CH_2-N-CH_2-, piperazine); 3.66 (s, 3H, -OCH_3); 5.37 (s, 2H, -CH_2-); 6.87 (m, 4H, Ar-H); 7.27 (d, 2H, *J* = 7.5 Hz, Ar-H); 7.64 (m, 4H, Ar-H); 7.69 (m, 1H, Ar-H); 8.42 (d, 1H, *J* = 7.5 Hz, Ar-H); 9.85 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.52, 154.62, 151.92, 141.04, 141.02, 136.30, 136.01, 134.39, 131.86, 130.41, 128.90 (2C), 128.04, 122.63, 122.48, 120.67, 119.02, 118.00, 111.66 (2C), 65.16, 50.12, 50.05 (2C), 40.30, 39.80; Analysis: calcd. for $C_{26}H_{26}N_5ClO$ (459.9): C 67.89, H 5.70, N 15.23%; found: C 67.59, H 5.58, N 14.99%.

1-[(4-(Pyrimidin-2-yl)piperazin-1-yl)metylo]-2-(2-chlorobenzylideneamino)benzimidazole (23)

Obtained as a yellow precipitate, yield 2.49 g (57%); crystallized from toluene; m.p. 164-170°C; IR (KBr, cm^{-1}): 3060 (CH arom.); 2950 (-CH_2-); 1600 (ring); 1550 (C=N); 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.64 (m, 4H, -CH_2-N-CH_2-, piperazine); 2.72 (m, 4H, -CH_2-N-CH_2-, piperazine); 5.34 (s, 2H, -CH_2-); 6.54 (m, 1H, Ar-H); 7.26 (m, 2H, Ar-H); 7.65 (m, 5H, Ar-H); 8.33 (m, 3H, Ar-H); 9.81 (s, 1H, -CH=N-); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 160.90, 160.66, 157.81, 154.56, 141.03, 136.28, 135.89, 134.35, 131.86, 130.38, 128.92, 128.01, 122.59 (2C), 119.02, 117.55, 109.98, 63.47, 49.77 (2C), 43.16 (2C); Analysis: calcd. for $C_{23}H_{22}N_7Cl$ (431.9): C 63.96, H 5.13, N 22.70%; found: C 63.61, H 4.90, N 22.04%.

1-[(Piperidin-1-yl)methyl]-2-(salicylamino)benzimidazole (24)

Obtained as a white precipitate, yield 2.08 g (62%); crystallized from ethanol; m.p. 159-161°C; IR (KBr, cm^{-1}): 3320 (NH), 3050 (CH, arom.), 2940, 2860 (-CH_2-), 1600 (ring), 1200 (C-O-H), 760 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.53 (m, 6H, -CH_2-CH_2-CH_2-, piperidine), 2.78 (m, 4H,

-CH_2-N-CH_2-, piperidine), 4.45 (d, 2H, *J* = 5.7 Hz, -CH_2-NH-), 5.13 (s, 2H, -CH_2-), 6.77 (m, 2H, Ar-H), 6.97 (m, 2H, Ar-*H*), 7.11 (H, Ar-H), 7.27 (m, 4H, -NH- and Ar-H), 9.72 (s, 1H, -OH); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 156.20, 147.66, 140.52, 138.65, 135.01, 128.93, 126.50, 124.35, 121.50, 120.76, 117.15, 116.90, 108.57, 63.84, 50.76 (2C), 42.04, 25.30 (2C), 23.40; Analysis: calcd. for $C_{20}H_{24}N_4O$ (336.43): C 71.40, H 7.19, N 16.65%; found: C 71.29, H 7.11, N 15.97%.

1-[(Morpholin-4-yl)methyl]-2-(salicylamino)benzimidazole (25)

Obtained as a white precipitate, yield 1.89 g (56%); crystallized from dioxane; m.p. 169-170°C; IR (KBr, cm^{-1}): 3350 (NH), 2920, 2860 (-CH_2-), 1600 (ring), 1580 (C=N), 1200 (C-O-H), 760 (CH, arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.87 (m, 4H, -CH_2-N-CH_2-, morpholine), 3.66 (m, 4H, -CH_2-O-CH_2-, morpholine), 4.47 (d, 2H, *J* = 6.0 Hz, -*CH_2*-NH-), 5.18 (s, 2H, -CH_2-), 6.78 (m, 2H, Ar-H), 6.97 (m, 2H, Ar-H), 7.10 (m, 1H, Ar-H), 7.29 (m, 4H, -NH- and Ar-H), 9.70 (s, 1H, -OH); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 155.34, 148.47, 140.59, 135.02, 134.25, 128.96, 126.40, 121.56, 120.91, 119.13, 117.15, 116.87, 108.53, 66.00 (2C), 63.14, 50.23 (2C), 42.58; Analysis: calcd. for $C_{19}H_{22}N_4O_2$ (338.40): C 67.44, H 6.55, N 16.56%; found: C 67.22, H 6.66, N 16,46%.

1-(Morpholin-1-yl)-methyl)-2-(4-hydroxybenzylamino)benzimidazole (26)

Obtained as a white precipitate, yield 0.95 g (28%); crystallized from dioxane; m.p. 164-165°C; IR (KBr, cm^{-1}): 3380 (NH), 2870 (-CH_2-), 1600 (ring), 1580 (NH), 1450 (C=N), 1140 (C-O-H), 820, 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.67 (m, 4H, -CH_2-N-CH_2-, morpholine); 3.54 (m, 4H, -CH_2-O-CH_2-, morpholine), 4.45 (d, 2H, *J* = 5.7 Hz, -*CH_2*-NH-), 5.29 (s, 2H, -CH_2-), 6.70 (d, 2H, *J* = 8.7 Hz, Ar-H), 6.92 (m, 3H, Ar-H), 7.21 (m, 4H, -NH- and Ar-H), 9.26 (s, 1H, -OH); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 156.48, 145.3, 141.52, 135.37, 131.25, 128.52, 128.36, 120.40, 120.37, 118.36, 114.95, 114.86, 108.08, 66.32, 66.14, 62.83, 51.57, 50.20, 45.29; Analysis: calcd. for $C_{19}H_{22}N_4O_2$ (338.40): C 67.84, H 5.99, N 16.66%; found: C 68.09, H 6.19, N 16.40%.

General procedure for the preparation of compounds 27-29

Reaction of 2-(4-hydroxybenzyl)aminobenzimidazole (**2**) with selected compound containing active methylene group: acetylacetone, benzoylacetone and malononitrile. To a solution of Schiff base **2** (0.01 mol) in ethanol (30 mL) containing triethylamine (0.3 mL) selected compounds containing active methylene group were added. The solution was refluxed for ca 8-10 h (TLC control). After cooling, the precipitate was filtered, washed with diethyl ether, dried and crystallized form appropriate solvent.

3-Acetyl-2-(4-hydroxyphenylene)-4-methyl-1,2-dihydropyrimido[1,2-a]benzimidazole (27)

Obtained as a white precipitate, yield 1.62 g (51%); crystallized from ethanol; m.p. 298-302°C; IR (KBr, cm^{-1}): 3230 (NH), 2950, 2860 (CH_3), 1600 (ring), 1500 (NH), 1340 (CH), 1230 (C-O-H), 1200, 860, 740 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 2.20 (s, 3H, -CH_3), 3.40 (s, 3H, -OCH_3), 6.50 (s, 1H, -NH-*CH*-), 6.64 (d, 2H, *J* = 8.7 Hz, Ar-H), 7.01 (m, 2H, Ar-H), 7.23 (d, 2H, *J* = 8.4 Hz, Ar-H), 7.33 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.41 (d, 1H, *J* = 8.4 Hz, Ar-H), 9.42 (s, 1H, -OH), 10.70 (s, 1H, -NH-, pyrimidine); ^{13}C NMR (300 MHz, DMSO, δ, ppm): 194.42, 158.93, 156.22, 142.31, 145.45, 132.03, 131.60, 128.64, 128.49 (2C), 121.61, 121,28, 119.97, 116.72, 115.13 (2C), 110.06, 30.43, 19.58; Analysis: calcd. for $C_{19}H_{17}N_3O_2$ (319.36): C 71.46, H 5.37, N 16.66%; found: C 71.43, H 5.42, N 16.27%.

3-Benzoyl-2-(4-hydroxyphenylene)-4-methyl-1,2-dihydropyrimido[1,2-a]benzimidazole (28)

Obtaind as a white precipitate, yield 1.57 g (41%); crystallized from ethanol; m.p. 291-294°C; IR (KBr, cm^{-1}): 3240 (NH), 2840 (CH_3), 1665 (C=O), 1230 (C-O-H), 835, 740 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 1.82 (s, 3H, -CH_3), 6.49 (s, 1H, -NH-*CH*-), 6.61 (d, 2H, *J* = 8.4 Hz, Ar-H), 6.90 (t, 1H, *J* = 7.2 Hz, Ar-H), 7.04 (t, 1H, *J* = 7.2 Hz, Ar-H), 7.10 (d, 1H, *J* = 8.4 Hz, Ar-H), 7.35 (d, 3H, *J* = 7.8 Hz, Ar-H), 7.48 (m, 5H, Ar-H), 9.38 (s, 1H, -OH), 10.62 (s, 1H, -NH-, pyrimidine); Analysis: calcd. for $C_{24}H_{19}N_3O_2$ (381.43): C 70.72, H 5.28, N 17.10%; found: C 70.45, H 5.16, N 17.67%.

4-Amino-3-cyano-2-(4-hydroxyphenylene)-1,2-dihydropyrimido[1,2-a]benzimidazole (29)

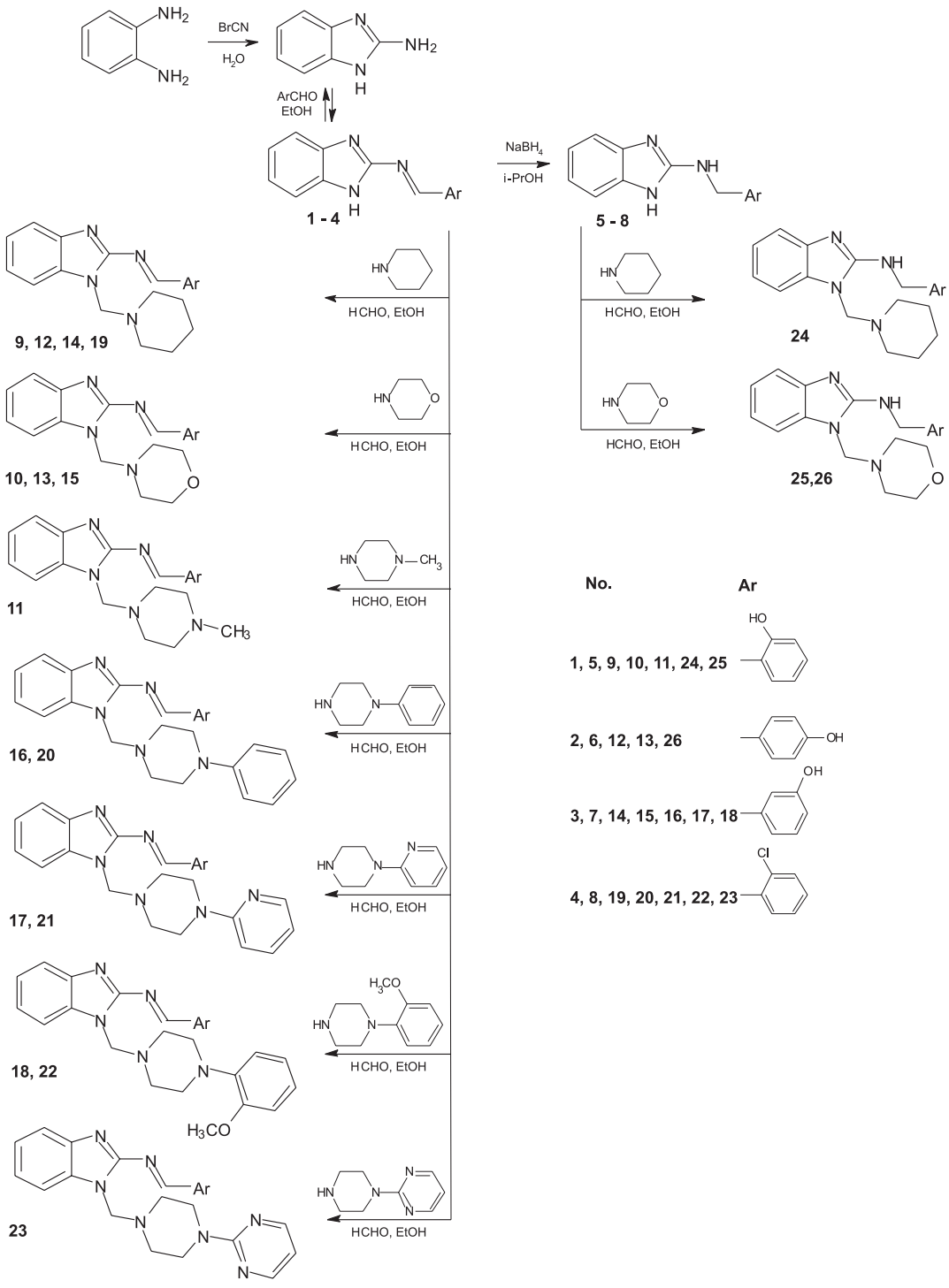
Obtained as a white precipitate, yield 1.62 g (54%); crystallized from butanol; m.p. 223-225°C; (KBr, cm^{-1}): 3500, 3400 (NH_2), 3350 (NH), 2900 (CH), 2200 (CN), 1260 (C-O-H), 830, 760 (CH arom.); ^{1}H NMR (300 MHz, DMSO, δ, ppm): 5.09 (s, 1H, -CH-), 6.73 (m, 4H, -NH_2 and Ar-H), 6.98 (d, 1H, *J* = 7.8 Hz, Ar-H), 7.10 (m, 3H, Ar-H); 7.21 (d, 1H, *J* = 7.2 Hz, Ar-H), 7.63 (d, 1H, *J* = 7.8 Hz, Ar-

H), 8.46 (s, 1H, -NH-, pyrimidine), 9.46 (s, 1H, -OH); Analysis: calcd. for $C_{17}H_{13}N_5O_1$ (303.32): C 67.51, H 5.67, N 22.21%; found: C 67.02, H 5.32, N 22.43%.

Biology

In vitro antiproliferative assay

Antiproliferative tests were performed on human cancer cell lines: A549 (lung), T47D



Scheme 1. Synthesis of Mannich bases

(breast), leukemia MV4-11 and mouse embryonic fibroblast BALB/3T3 according to standard procedure (33). All cell lines were obtained from American Type Culture Collection (Rockville, Maryland, USA) and have been maintained in culture or frozen in thaw Cell Culture Collection of the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (IIET, PAS, Wrocław, Poland). The A549 and T47D cells were cultured in a mixture of Opti-MEM and RPMI 1640 medium (1 : 1, both from Gibco, Scotland, UK) supplemented with 2 mM L-glutamine and 5% fetal bovine serum. The culture of T47D cells were supplemented with 0.8 mg/L of insulin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). MV4-11 cells were cultured in RPMI 1640 medium (Gibco, Scotland, UK) with 2 mM L-glutamine, adjusted to contain 1.5 g/L sodium bicarbonate and 1.0 mM sodium pyruvate, 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Mouse fibroblasts BALB/3T3 were maintained in Dulbecco medium (DMEM, Gibco, Scotland, UK) supplemented with 2 mM L-glutamine and 10% fetal bovine serum (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). All culture media were supplemented with 100 units/mL penicillin, and 100 μg/mL streptomycin (Polfa Tarchomin S.A., Warszawa, Poland). Cell lines were grown at 37°C with 5% CO_2 humidified atmosphere. The anti-proliferative effect of the tested compound was examined after 72 h exposure of the cultured cells to varying concentrations of the test compound (total plate incubation time: 96 h), using the sulforhodamine B (SRB) assay for adherent cells (A549, BALB/3T3 and MCF-7) and MTT assay for leukemia cells (MV4-11) (33). The results were shown as an IC_{50} value (inhibitory concentration 50% - a concentration in μg/mL of tested agent which inhibits proliferation of 50% of cancer cells population). Each compound was tested at every concentration in triplicate in a single experiment, which was repeated 3 times. The activity of tested compound was compared to the activity of cisplatin, used as a reference agent.

RESULTS AND DISCUSSION

Chemistry

Schiff bases **1-4** have been obtained in the reactions of 2-aminobenzimidazole with selected aromatic aldehydes: salicylic-, 4-hydroxy, 3-hydroxy, 2-chloro- (32) benzaldehyde (Scheme 1). The reactions were carried out in boiling ethanol with the presence of catalytic amounts of Triflate. Schiff bases **1-4** formed crystals of different shades of yellow due to the presence of chromophoric groups (CH=N) in their molecules. The product structures have been confirmed by elemental analysis and IR, ^1H and ^{13}C NMR spectra.

IR spectra of Schiff bases contain, among other absorption bands, those in the range of ν = 1635-1680 cm^{-1} characteristic for the chain groups C=N. The presence of CH=N proton was confirmed by ^1H NMR spectra of all imines in which one-proton singlets at δ = 9.31-9.67 ppm were observed. One-proton singlets at δ = 12.51-12.73 ppm were assigned to the imidazole group NH. For compounds **1-3** one-proton singlets at δ = 9.87-12.13 ppm characteristic for -OH group were observed. The signals corresponding to aromatic protons were observed in the range of δ = 7.01-7.92 ppm.

Azomethine bond (–N=CH-) in imines **1-4** have been subjected to selective reduction using $NaBH_4$ in boiling *i*-propanol. The extent of the hydrogenation has been monitored by TLC and decoloring of yellow solution. In ^1H NMR spectra of 2-benzylaminobenzimidazoles **5-8** the absence of one-proton singlets at δ = 9.31-9.67 ppm were observed, whereas, two-proton doublets at $\delta \sim 4.50$ ppm ($J \sim 5.50$ Hz) ascribed to NH-C**H**$_2$ protons are present. Broad one-proton signal at $\delta \sim 7.10$ ppm was ascribed to N**H**-CH_2 protons. Other signals of protons are observed in the similar places like in Schiff bases.

In the next stage of our work, Schiff bases **1-4** and 2-benzylaminobenzimidazole derivatives **5-8** were used as substrates for the Mannich condensation with, selected, pharmacophore, secondary amines: morpholine, piperidine, N-methylpiperazine, N-phenylpiperazine, 1-(2-pyridyl)piperazine, 1(2-methoxyphenyl)piperazine, 1-(2-pyrimidinyl) piperazine and formaldehyde. For the synthesis we have chosen amines that were used in the Mannich condensations and gave active antiproliferative compounds (12-23). The reactions were carried out in ethanol at room temperature. Under these reaction conditions, only the compounds presented in Scheme 1 have been obtained.

In ^1H NMR spectra of compounds **9-23** two-proton singlets at $\delta \sim 5.25$ ppm characteristic for -CH_2- group were observed, instead of one-proton singlet of NH imidazole. In case of 2-benzylaminobenzimidazoles **5-8** there are two possible aminomethylation paths: one in the position 1 or 2 or second at 1 and 2 positions. The elementary analyses confirm one aminomethylene group in obtained compounds **24-26**. In ^1H NMR spectra the absence of signal characteristic for NH imidazole was observed. Two-proton singlets at $\delta \sim 5.15$ ppm from -CH_2- group were assigned.

Another way of chemical modification of Schiff base **2** were reactions with selected compounds containing active methylene group; 1,3-diketones: acetyl-, benzoylacetone, or malononitrile in boiling ethanol with catalyctic amounts of triethylamine (Scheme 2). Tricyclic 2-(4-hydroxybenzylidene)-aminobenzimidazole (**2**) derivatives were obtained. In the first step of the reaction an unstable adducts **A** and **B** were formed. Elementary analysis confirmed that elimination of one molecule of water form adducts **A** lead to pyrimido[1,2-a]benzimidazole derivatives **27**, **28**. Cyclization of unstable adduct **B** gave 4-amino-3-cyano-2-(4-hydroxyphenylene)-1,2-dihydropyrimido[1,2-*a*]benzimidazole (**29**).

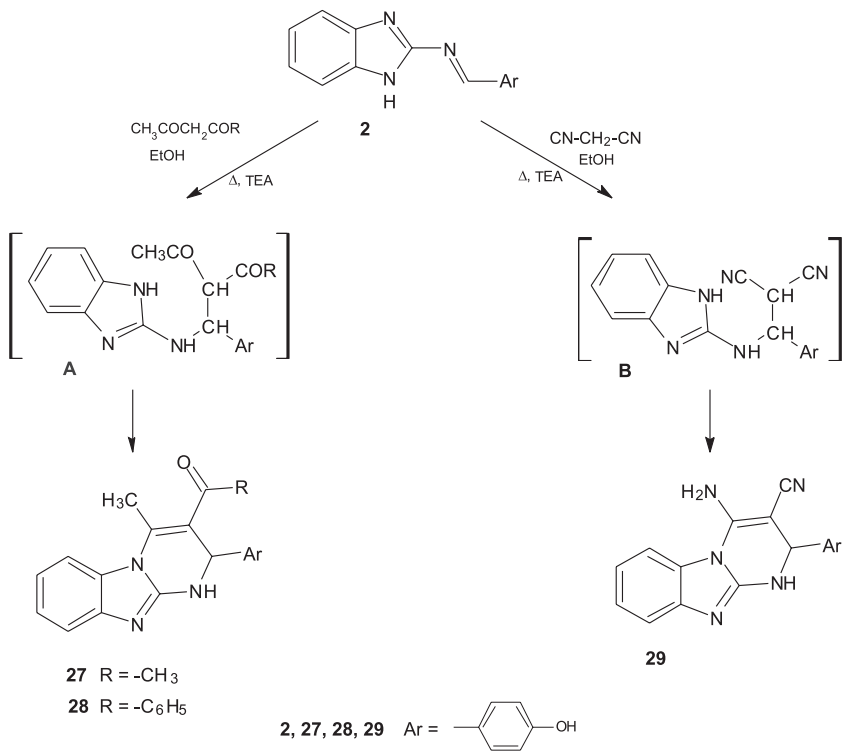
In the ^{1}H NMR spectra of 3-acetyl- (**27**), 3-benzoyl-2-(4-hydroxyphenylene)-4-methyl-1,2-dihydropyrimido[1,2-*a*]benzimidazole (**28**) and 4-amino-3-cyano-2-(4-hydroxyphenylene)-1,2-dihydropyrimido[1,2-a]benzimidazole (**29**) are displayed two one proton singlet signals at $\delta \sim 6.50$ ppm and $\delta \sim 10.65$ ppm or at $\delta = 5.09$ ppm and $\delta = 8.46$ ppm which were assigned to the -C**H**-N**H**- protons, respectively. Mutual splitting of signals was not recorded because of fast protons exchange from N**H** group.

Number of signals for the aromatic protons in the ^{1}H NMR spectra of all compounds is in good agreement with their structures.

Twenty seven **1-3**, **5-29** new compounds of various chemical structures were obtained from the syntheses described here. These derivatives may also be used as starting materials for further syntheses. All synthesized compounds were screened for their antiproliferative activity *in vitro* against the cells of 4 human tumor cell lines. The obtained compounds seem to be suitable candidates for further chemical modifications and might be of interest as pharmacologically active compounds.

Biological activity

To screen the antiproliferative activity of the compounds, the cell lines of various origin: leukemia, breast, lung cancer have been chosen. Selected types of cancer are the examples of diseases frequently afflicted people worldwide. Moreover, we have performed the comparative evaluation of cytotoxicity on mouse fibroblast cell line BALB/3T3. This cell line is recommended by various agencies introducing alternative methods for testing toxicity of compounds. For example, these



Scheme 2. Reaction of 2-(4-hydroxybenzylideneamino)benzimidazole with compounds containing active methylene group

cells were used in some methods aiming to estimate starting doses for oral acute systemic toxicity of compounds under the European Center of Validation of Alternative Methods (ECVAM) guidelines (OECD guidance document (GD) 129 published in 2010).

The synthesized compounds were screened for their antiproliferative activity using cells of MV4-11 human biphenotypic B myelomonocytic leukemia (Table 1). In comparison to cisplatin, the activity of tested compounds was lower, however we selected some compounds with IC_{50} value ranged between 0.23-4.25 μg/mL for further studies on the cells of breast and lung cancer, as well as on normal mouse fibroblasts to assess their selectivity towards cancer cells.

The antiproliferative activity *in vitro* of selected compounds **5**, **7**, **9-16**, **24-26**, **28**, **29** was significantly lower against lung and breast cancer cells, comparing to the results obtained against MV4-11 cells. However, one of them showed interesting profile of activity. Namely, the activity of compound **29** towards mouse fibroblasts was significantly lower than towards cancer cells, suggesting low toxicity (Table 2). Particularly, the most active on all cancer cells compound **29**, was inactive against normal fibroblasts.

The results revealed that all screened compounds demonstrated good to promising antiproliferative activity *in vitro* against MV4-11 human leukemia cell line. Among Schiff bases **1-4** the highest anticancer activity shows 2–(2-chlorobenzylideneamino)-1*H*-benzimidazole (**4**), containing in his structure 2-chlorophenylene substituent. Selective reduction of azomethine bond in Schiff bases **1-4** provides 2-benzylaminobenzimidazoles **5-8**, which were more active than the substrates. The presence of chlorine group in aromatic ring increases the anticancer activity, whereas the hydroxyl group decreases it. The most active compound was 2-(2-chlorobenzylamino)-1*H*-benzimidazole (**8**). All obtained Mannich bases **9-26** showed good antiproliferative activity *in vitro*. The promising activity possesses 2-salicylideneamino-1*H*-benzimidazoles **9-11** and 3-hydroxyamino-1*H*-benzimidazole **14**, substituted in 1 position with: piperidine, morpholine and 4-methylpiperazine. Comparable activity was shown by 1-[(piperidin-1-yl)methyl]- (**24**), 1-[(morpholin-4-yl)methyl]-2-(salicylamino)benzimidazole (**25**) and 1-(morpholin-1-yl)-methyl)-2-(4-hydroxybenzylamino)benzimidazole (**26**).

Among series of tricyclic 2-(4-hydroxyphenylene)pyrimido[1,2-*a*]benzimidazole derivatives the highest antiproliferative activity possesses compound **29**, substituted in position 3 and 4 with cyano and amino group, respectively. Replacement of cyano group in 3 position with benzoyl and amino group in position 4 with methyl decreased 10 times the antiproliferative activity. 3-Acetyl-2-(4-hydroxyphenylene)-4-methyl-1,2-dihydropyrimido[1,2-

Table 1. The antiproliferative activity of compounds against the cells of MV4-11 human leukemia cell line.

Compound	IC_{50} [mg/mL] mean ± SD	Compound	IC_{50} [mg/mL] mean ± SD
1	14.47 ± 0.96	**16**	3.40 ± 0.14
2	28.10 ± 2.77	**17**	4.80 ± 1.37
3	18.64 ± 2.23	**18**	4.34 ± 0.55
4	4.33 ± 4.87	**19**	3.31 ± 0.1
5	2.41 ± 0.58	**20**	6.67 ± 3.87
6	5.52 ± 0.11	**21**	4.72 ± 0.74
7	3.83 ± 0.55	**22**	5.88 ± 2.13
8	1.88 ± 1.42	**23**	4.75 ± 2.66
9	2.32 ± 0.11	**24**	2.48 ± 0.60
10	2.56 ± 0.53	**25**	3.07 ± 0.61
11	2.13 ± 0.28	**26**	2.41 ± 0.18
12	4.20 ± 1.78	**27**	n.a.
13	4.25 ± 1.2	**28**	2.65 ± 0.47
14	2.84 ± 0.31	**29**	0.23 ± 0.05
15	3.08 ± 0.24	Cisplatin	0.04 ± 0.01

n.a. = not active in the range of concentrations used

Table 2. The antiproliferative activity of selected compounds against human breast (T47D) and lung (A549) cancer and normal mouse fibroblasts (BALB/3T3) cell lines.

Compound	Cel line/ IC_{50} mg/mL		
	T47D	A549	BALB/3T3
5	9.55 ± 1.26	24.9 ± 1.71	38.10 ± 2.31
7	23.19 ± 1.97	36.62 ± 1.88	n.a.
9	24.55 ± 1.48	37.10 ± 0.96	49.27 ± 3.82
10	18.52 ± 3.79	36.59 ± 0.25	44.05 ± 3.26
11	20.92 ± 4.55	35.16 ± 1.98	38.01 ± 1.53
12	38.12 ± 3.51	36.16 ± 0.66	43.91 ± 1.67
13	38.59 ± 1.73	36.45 ± 2.71	36.68 ± 1.59
14	39.75 ± 0.81	34.94 ± 2.91	41.61 ± 2.28
15	33.11 ± 2.29	34.67 ± 2.39	38.42 ± 2.03
16	35.33 ± 3.29	39.61 ± 0.90	44.49 ± 1.63
24	15.83 ± 1.00	33.31 ± 1.18	40.23 ± 2.88
25	17.83 ± 0.44	31.60 ± 1.00	45.29 ± 2.76
26	24.95 ± 1.70	35.29 ± 2.12	38.59 ± 2.61
28	n.a.	n.a.	n.a.
29	6.69 ± 0.24	5.72 ± 0.81	n.a.
cisplatin	2.78 ± 0.55	2.40 ± 0.64	3.04 ± 0.83

n.a. = not active in the range of concentrations used

a]benzimidazole (**27**) was inactive in the range of concentrations used.

It is difficult, on the basis of obtained results, to make a broader SAR discussion.

CONCLUSIONS

Twenty seven new compounds **1-3**, **5-29** of various chemical structure: Schiff bases, 2-benzylaminobenzimidazole, Mannich bases and pyrimido[1,2-*a*]benzimidazole derivatives were obtained by the syntheses described here. Their structures were confirmed by elemental analysis and IR, ^{1}H and ^{13}C NMR spectra. All synthesized compounds **1-29** were screened for their antiproliferative activity *in vitro* on MV4-11 human leukaemia cell line. The results revealed that all screened compounds demonstrated good to promising antiproliferative activity against human leukemia cell line. The most active compounds were then tested towards human breast T47D and lung A549 cancer cell lines and normal mouse fibroblasts.

The most active compound against the cells of cancer cell lines was 4-amino-3-cyano-2-(4-hydroxyphenylene)-1,2-dihydropyrimido[1,2-*a*]benzimidazole (**29**) (IC_{50} 0.23 ± 0.05 μg/mL against MV4-11 cells) showing in parallel very low cytotoxicity towards mouse fibroblasts. Cisplatin was used as reference drug (IC_{50} 0.04 ± 0.01 μg/mL).

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NATURAL DRUGS

NEW ALIPHATIC ESTER, β-SITOSTEROL DIGLUCOSIDE AND VESICARIA BIFLAVONES FROM THE SEEDS OF *RUMEX VESICARIUS* L.

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Abstract: *Rumex vesicarius* L. (Polygonaceae) is an annual, monoecious, glabrous, pale green herb cultivated as a leafy vegetable in south western Asia and northern Africa. Its seeds are prescribed as a refrigerant, laxative, antidote for scorpion venom and to cure dysentery and liver diseases. Phytochemical investigation of a methanolic extract of the seeds of *R. vesicarius* resulted in the isolation of a new aliphatic ester *n*-heptacosanyl *n*-hexanoate (**2**), a steroidal diglucoside stigmasta-5-en-3-ol-3-*O*-β-D-glucopyranosido-(4→1'')-*O*-β-D-glucopyranoside (**3**) and two bioflavonoids characterized as (2a,3a-*trans*)-3a(β),5a,7a,3'a,4'a-pentahydroxyflavanolyl-(8a→2')-5,7,3'-trihydroxy-4'-methoxy-8-*n*-but-3''-enyl-flavanone (**4**) and 5,7,3',4',5'-pentahydroxy-8-(*cis*-1''α,2''β-dihydroxyhept-4''-enyl-7''-oic acid)-flavanoyl-(2'→8a)-5a,7a,3'a,5'a-tetrahydroxy-4'a-methoxyflavanone (**5**) together with stigmasterol (**1**). The structures of all the isolated phytoconstituents have been established on the basis of spectral data analysis and chemical reactions.

Keywords: *Rumex vesicarius*, Polygonaceae, seeds, aliphatic ester, β-sitosterol diglucoside, biflavonoids, *c*haracterization

Rumex vesicarius L. (Polygonaceae), known as Chukra or Bladder dock, is an annual, monoecious, glabrous, dichotomously branched, succulent pale green herb. It is a native to south western Asia and northern Africa; cultivated as a leafy vegetable in many parts of India (1). It is prescribed to treat asthma, bronchitis, constipation, calculus, dyspepsia, flatulence, hepatic diseases, heart troubles, hiccough, indigestion, nausea pains, spleen diseases, piles, scabies, leucoderma, toothache and tumors (1-4). It possesses diuretic, antiscorbutic, appetizer, astringent, carminative, laxative, stomachic and tonic properties. The leaves are eaten fresh and much appreciated for their acid taste; they can be added to salads and used as an antidote for snake venom. The plant is prescribed to reduce biliary disorders and to control cholesterol levels. The seeds are utilized as a refrigerant, to cure dysentery and as an antidote for scorpion venom. The seed powder is taken orally to treat liver diseases and as a laxative (1-3). The plant contained flavonoids (vitexin, isovitexin, orientin and isorientin), anthraquinones, particularly in roots (emodin and chrysophanol), quinones, carotenoids, vitamins, proteins, lipids, carbohydrates, reducing sugars, phenols, tannins, saponins, triterpenoids and organic acids (5-10). The drug showed antidiarrheal and antidysenteric (10), antimicrobial (9, 11-13), antioxidant (14) and diuretic (15) activities. The present paper describes the isolation and characterization of four new phytoconstituents from the seeds of *R. vesicarius*.

EXPERIMENTAL

General

Melting points were determined on a Perfit melting apparatus (Ambala, Haryana, India) and are uncorrected. UV spectra were measured with a Lambda Bio 20 spectrophotometer (Perkin-Elmer-Rotkreuz, Switzerland) in methanol. Infra red spectra were recorded on Bio-Rad FTIR 5000 (FTS 135, Kawloon, Hong Hong) spectrophotometer using KBr pellets; ν_{max} values are given in cm^{-1}. ^{1}H and ^{13}C NMR spectra were scanned on Advance DRX Bruker spectrospin 400 and 100 MHz, respectively, instruments (Karlsruhe, Germany) using TMS as an internal standard. Mass spectra were obtained by effecting FAB ionization at 70 eV on a JEOL-JMS-

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DX 303 spectrometer (Japan) equipped with direct inlet probe system. Column chromatography was performed on silica gel (60-120 mesh; Qualigen, Mumbai, India). TLC was run on silica gel G (Qualigen). Spots were visualized by exposing to iodine vapors, UV radiation and spraying with ceric sulfate solution.

Plant material

The seeds of *R. vesicarius* were procured from the Khari Baoli market of Delhi and identified by Prof. M.P. Sharma, Department of Botany, Jamia Hamdard, New Delhi. A voucher specimen is deposited in the herbarium of the Phytochemical Research Laboratory, Faculty of Pharmacy.

Extraction and isolation

The air-dried seeds (2.0 kg) were coarsely powdered, defatted with petroleum ether and extracted with methanol exhaustively in a Soxhlet apparatus. The combined extracts were filtered and concentrated under reduced pressure to get a dark brown viscous mass (125 g, 6.25%). The dried extract was dissolved in minimum quantity of methanol and adsorbed on silica gel (60-120 mesh) for preparation of a slurry. It was dried in air and chromatographed over silica gel column (1.6 m × 16 mm × 2 mm) packed in petroleum ether. The column was eluted successively with different solvents in increasing order of polarity in various combinations of chloroform, chloroform-methanol (19.9 : 0.1; 99 : 1; 97 : 3; 19 : 1; 93 : 7; 9 : 1; 17 : 3; 3 : 1; 3 : 2; 2 : 3, v/v) and methanol. The fractions were collected separately and matched by TLC to check homogeneity. Similar fractions having the same R_f values were combined and crystallized. The isolated compounds were recrystallized to get pure compounds. The following compounds were isolated from the methanolic extract of the seeds of *R. vasicarius:*

Stigmasterol (1)

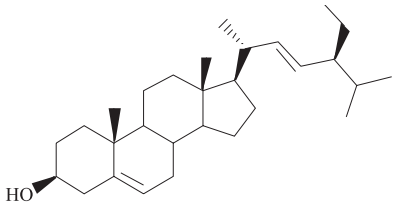
Elution of the column with chloroform gave a colorless, amorphous powder of **1**, recrystallized from acetone, 50 mg (0.0025% yield); R_f: 0.51 (petroleum ether-$CHCl_3$-MeOH, 1 : 4 : 1, v/v/v); co-TLC comparable; m.p. and m.m.p.: 168-170°C; IR (KBr, cm^{-1}): 3480, 2930, 2853, 1640, 1470, 1260, 1180, 1020; ^{1}H NMR ($CDCl_3$, δ, ppm): 5.35 (brs, 1H, H-5), 5.14 (dd, 1H, *J* = 15.3, 8.4 Hz, H-22), 5.02 (dd, 1H, *J* = 8.1, 15.3 Hz, H-23), 3.95 (brm, 1H, $w_{1/2}$ = 18.5 Hz, H-3α), 1.01 (brs, 3H, Me-19), 0.93 (d, 3H, *J* = 6.3 Hz, Me-21), 0.84 (d, 3H, *J* = 6.3 Hz, Me-26), 0.82 (d, 3H, *J* = 6.3 Hz, Me-27), 0.80 (d, 3H, *J* = 6.2 Hz, Me-29), 0.69 (brs, 3H, Me-18); +ve ESI MS *m/z*: 412 $[M]^+$ ($C_{29}H_{48}O$) (8.2).

***n*-Heptacosanyl *n*-hexanoate (2)**

Further elution of the column with chloroform eluants produced colorless crystals of **2**, recrystallized from methanol, 157 mg (0.078% yield); R_f: 0.40 ($CHCl_3$-MeOH, 4 : 1, v/v); m.p.: 70-72°C; IR (KBr, cm^{-1}): 2920, 2852, 1718, 1635, 1465, 1380, 1225, 1165, 785; ^{1}H NMR ($CDCl_3$, δ, ppm): 4.17 (t, 2H, *J* = 6.8 Hz, H_2-1'), 2.31 (t, 2H, *J* = 7.2 Hz, H_2-2), 2.01 (m, 2H, H_2-3), 1.68 (m, 2H, CH_2), 1.25 (brs, 52H, 26 × CH_2), 0.87 (t, 3H, *J* = 6.5 Hz, Me- 27'), 0.83 (t, 3H, *J* = 6.1 Hz, Me-6); ^{13}C NMR ($CDCl_3$, δ, ppm): 173.74 (C-1), 74.09 (C-1'), 39.18 (CH_2), 37.30 (CH_2), 34.77 (CH_2), 34.17 (CH_2), 32.45 (CH_2), 31.95 (CH_2), 30.06 (CH_2), 29.72 (CH_2), 29.68 (10 × CH_2), 29.61 (CH_2), 29.56 (CH_2), 29.39 (CH_2), 29.34 (CH_2), 29.22 (CH_2), 29.18 (CH_2), 28.97 (CH_2), 27.99 (CH_2), 25.34 (CH_2), 25.22 (CH_2), 22.71 (CH_2), 14.19 (C-27'), 14.13 (C-6); +ve ESI MS *m/z*: 494 $[M]^+$ ($C_{33}H_{66}O_2$) (5.1), 395 (11.7).

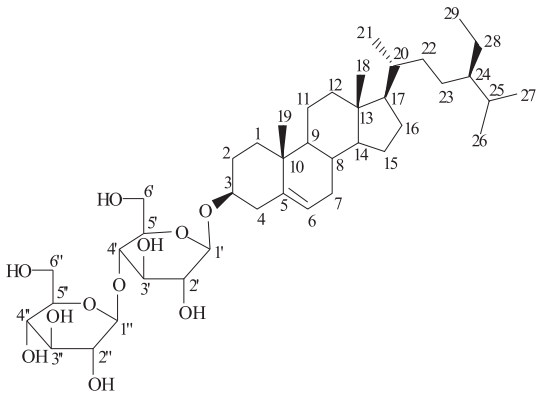
β-Sitosterol diglucoside (3)

Elution of the column with chloroform-methanol (19 : 1, v/v) afforded a colorless, amorphous powder of **3**, recrystallized from methanol, 55 mg (0.003% yield); R_f: 0.67 ($CHCl_3$-MeOH, 1 : 1, v/v); m.p.: 225°C; IR (KBr, cm^{-1}): 3450, 3404, 3380, 2919, 2848, 1645, 1474, 1100, 1080; UV λ_{max} (MeOH): 243 nm (log ε 3.2); ^{1}H NMR (DMSO-d_6, δ, ppm): 5.55 (d, 1H, *J* = 5.1 Hz, H-6), 5.32 (d, 1H, *J* = 7.8 Hz, H-1'), 4.90 (d, 1H, *J* = 7.7 Hz, H-1''), 4.48 (m, 2H, H-5', H-5''), 4.23 (m, 1H, H-2'), 4.13 (m, 1H, H-2''), 4.01 (m, 1H, H-3'), 3.81 (m, 1H, H-3''), 3.54 (brm, 1H, $w_{1/2}$ = 18.3 Hz, H-3α), 3.50 (m, 1H, H-4'), 3.45 (m, 1H, H-4''), 3.18 (brs, 2H, H_2-6)', 3.07 (brs, 2H, H_2-6''), 0.99 (brs, 3H, Me-19), 0.95 (d, 3H, *J* = 6.1 Hz, Me-21), 0.88 (d, 3H, *J* = 5.8 Hz, Me-27), 0.84 (d, 3H, *J* = 6.0 Hz, Me-26), 0.82 (d, 3H, *J* = 6.1 Hz, Me-29), 0.65 (brs, 3H, Me-18); ^{13}C NMR ($CDCl_3$, δ, ppm): 36.84 (C-1), 32.11 (C-2), 73.46 (C-3), 41.49 (C-4), 140.45 (C-5), 121.18 (C-6), 31.35 (C-7), 34.36 (C-8), 49.63 (C-9), 36.18 (C-10), 20.60 (C-11), 39.79 (C-12), 41.49 (C-13), 56.19 (C-14), 24.50 (C-15), 28.73 (C-16), 55.43 (C-17), 13.92 (C-18), 18.68 (C-19), 35.49 (C-20), 22.11 (C-21), 33.37 (C-22), 25.47 (C-23), 45.16 (C-24), 29.07 (C-25), 18.73 (C-26), 19.02 (C-27), 23.86 (C-28), 11.68 (C-29), 103.48 (C-1'), 70.09 (C-2'), 70.58 (C-3'), 68.32 (C-4'), 76.76 (C-5'), 61.09 (C-6'), 100.81 (C-1''), 70.79 (C-2''), 70.16 (C-3''), 65.75 (C-4''), 76.71 (C-5''), 60.08 (C-6''); +ve ESI MS m/z (rel. int.): *m/z* 738 $[M]^+$ ($C_{41}H_{70}O_{11}$) (2.6), 413 (21.3)

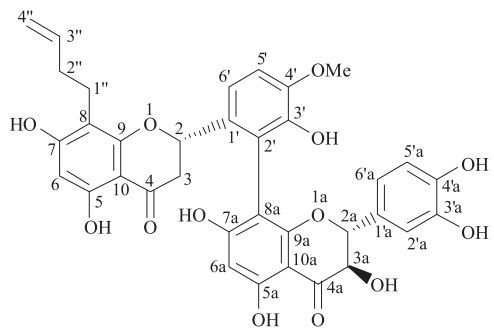


1. Stigmasterol

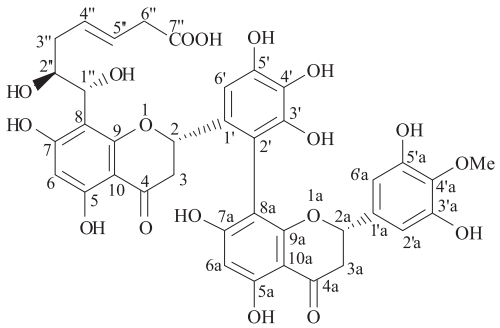
2. *n*-Heptacosanyl-*n*-hexanoate



3. β-Sitosterol diglucoside



4. Vesicariabiflavanone A



5. Vesicariabiflavanone B

Figure 1. Structures of compounds **1-5** isolated from the methanolic extract of the seeds of *Rumex vesicarius* L.

($C_{29}H_{49}O$) (3.7), 398 (11.9), 273 (9.8), 255 (33.7), 240 (25.8), 213 (14.1).

Vesicariabiflavanone A (4)

Further elution of the column with chloroform-methanol (19 : 1, v/v) yielded a yellow, amorphous powder of **4**, recrystallized from methanol, 286 mg (0.0143% yield); R_f: 0.55 ($CHCl_3$-MeOH, 1 : 1, v/v); m.p.: 203-205°C; IR (KBr, cm^{-1}): 3410, 3380, 3310, 2925, 2855, 1705, 1695, 1640, 1596, 1575, 1470, 1380, 1120, 1060; UV λ_{max} (MeOH): 255, 288, 370 nm (log ε 0.8, 5.3, 1.3); ^{1}H NMR ($CDCl_3$, δ, ppm): 6.94 (brs, 1H, H-6), 6.83 (brs, 1H, H-6a), 6.76 (d, 1H, *J* = 7.8 Hz, H-5'), 6.72 (d, 1H, *J* = 7.5 Hz, H-5'a), 6.70 (d, 1H, *J* = 2.5 Hz, H-2'a), 6.68 (d, 1H, *J* = 7.8 Hz, H-6'), 6.65 (dd, 1H, *J* = 2.5, 7.5 Hz, H-6'a), 5.25 (brm, 1H, H-3''), 5.07 (d, 1H, *J* = 5.8 Hz, H_2-4''a), 5.04 (d, 1H, *J* = 5.8 Hz, H_2-4''b), 4.98 (dd, 1H, *J* = 13.1, 2.9 Hz, H-2), 4.95 (d, 1H, *J* = 11.1 Hz, H-2a), 4.48 (d, 1H, *J* = 11.1 Hz, H-3a), 3.31 (brs, 3H, OMe), 3.11 (dd. 1H, *J* = 13.1, 17.2 Hz, H_2-3*ax*), 2.93 (dd, 1H, *J* = 2.9, 17.2 Hz, H_2-3*eq*), 2.73 (brm, 2H, H_2-1''), 2.61 (brm, 2H, H_2-2''); ^{13}C NMR ($CDCl_3$, δ, ppm): 82.57 (C-2), 42.06 (C-3), 196.04 (C-4), 163.97 (C-5), 95.79 (C-6), 166.49 (C-7), 94.62 (C-8), 162.98 (C-9), 101.40 (C-10), 129.47 (C-1'), 114.26 (C-2'), 144.33 (C-3'), 144.73 (C-4'), 114.69 (C-5'), 118.78 (C-6'), 44.53 (C-1''), 39.04 (C-2''), 129.03 (C-3''), 102.47 (C-4''), 80.51 (C-2a), 71.39 (C-3a), 194.90 (C-4a), 163.08 (C-5a), 95.57 (C-6a), 166.10 (C-7a), 92.53 (C-8a), 162.22 (C-9a), 99.70 (C-10a), 129.02 (C-1a'), 113.06 (C-2a'), 144.02 (C-3a'), 144.77 (C-4a'), 114.61 (C-5a'), 117.18 (C-6a'), 55.04 (OMe); +ve ESI MS *m*/*z* (rel. int.): 658 $[M]^+$ ($C_{35}H_{30}O_{13}$) (1.2), 356 $[C_{20}H_{20}O_6]^+$ (2.1), 302 $[C_{15}H_{10}O_7]^+$ (11.2), 152 (14.3).

Vesicariabiflavanone B (5)

Further elution of the column with chloroform-methanol (19 : 1, v/v) furnished a yellow, amorphous powder of **5**, recrystallized from methanol, 519 mg (0.0259% yield); R_f: 0.65 ($CHCl_3$-MeOH, 1 : 1); m.p.: 210°C; IR (KBr, cm^{-1}): 3485, 3404, 3380, 2955, 1695, 1685, 1640, 1550, 1410, 1320, 1250, 1065, 960, 855; UV λ_{max} (MeOH): 242, 291, 366 nm (log ε 1.1, 5.6, 1.3); ^{1}H NMR (DMSO-d_6, δ, ppm): 6.96 (s, 1H, H-6), 6.88 (s, 1H, H-6a), 6.76 (s, 1H, H-6'), 6.71 (d, 1H, *J* = 2.5 Hz, H-2'a), 6.67 (d, 1H, *J* = 2.5 Hz, H- 6'a), 5.76 (m, 1H, $w_{1/2}$ = 8.5 Hz, H-4''), 5.36 (m, 1H, $w_{1/2}$ = 8.3 Hz, H-5''), 5.28 (dd, 1H, *J* = 12.8, 2.9 Hz, H-2), 5.16 (dd, 1H, *J* = 12.6, 2.7 Hz, H-2a), 4.51 (d, 1H, *J* = 6.3 Hz, H-1''), 3.71 (m, 1H, $w_{1/2}$ = 14.7 Hz, H-2''α), 3.15 (brs, 3H, OMe), 3.12 (dd, 1H, *J* = 17.2, 12.8 Hz, H_2-3*ax*), 3.08 (dd, 1H, *J* = 17.3, 12.7 Hz, H_2-3'*ax*), 2.95 (d, 1H, *J* = 15.6 Hz, H_2-6''a), 2.90 (d, 1H, *J* = 15.6 Hz, H_2-6''b), 2.85 (dd, 1H, *J* = 2.9, 17.2 Hz, H_2-2*eq*), 2.80 (dd, 1H, *J* = 2.7, 17.3 Hz, H_2-2*eq*), 2.64 (m, 1H, H_2-3''a), 2.48 (m, 1H, H_2-3''b); ^{13}C NMR ($CDCl_3$, δ, ppm): 78.59 (C-2), 44.92 (C-3), 197.85 (C-4), 164.45 (C-5), 96.18 (C-6), 166.93 (C-7), 95.14 (C-8), 163.63 (C-9), 101.96 (C-10), 130.12 (C-1'), 115.51 (C-2'), 145.32 (C-3'), 145.88 (C-4'), 144.47 (C-5'), 114.47 (C-6'), 83.21 (C-1''), 71.73 (C-2''), 38.21 (C-3''), 128.22 (C-4''), 127.23 (C-5''), 115.32 (C-6''), 188.02 (C-7''), 78.20 (C-2a), 42.19 (C-3a), 196.41 (C-4a), 163.02 (C-5a), 95.96 (C-6a), 166.77 (C-7a), 93.33 (C-8a), 163.48 (C-9a), 100.63 (C-10a), 129.65 (C-1a'), 114.30 (C-2a'), 145.08 (C-3a'), 145.65 (C-4a'), 144.41 (C-5a'), 115.32 (C-6a'), 55.72 (OMe); +ve ESI MS *m*/*z*: 766 $[M]^+$ ($C_{37}H_{34}O_{18}$) (3.1), 317 (5.3), 166 (9.2).

RESULTS AND DISCUSSION

Compound **1** was the known phytoconstituent identified as stigmasterol (Fig. 1).

Compound **2** was obtained as colorless crystals from chloroform eluants. Its IR spectrum displayed important absorption bands for ester group (1718 cm^{-1}) and long aliphatic chain (785 cm^{-1}). Its mass displayed a molecular ion peak at *m*/*z* 494 corresponding to molecular formula of an ester $C_{33}H_{66}O_2$. An ion peak arising at *m*/*z* 395 [$C_{1'}$-O ester fission, $CH_3(CH_2)_{26}O]^+$ suggested that *n*-heptacosanyl moiety was esterified with *n*-hexanoic acid. The ^{1}H NMR spectrum of **2** displayed two two-proton triplets at δ 4.17 (*J* = 6.8 Hz) and 2.31 (*J* = 7.2 Hz) ppm assigned to oxygenated methylene H_2-1' protons and methylene H_2-2 protons adjacent to ester group, respectively. The remaining methylene protons appeared as two-proton multiplets at δ 2.01 and 1.68 ppm and as a broad singlet at δ 1.25 (28 × CH_2) ppm. Two three-proton triplets at δ 0.87 (*J* = 6.5 Hz) and 0.83 (*J* = 6.1 Hz) ppm were accounted to terminal primary C-27 and C-6´ methyl protons, respectively. The ^{13}C NMR of **2** exhibited signals for ester carbon at δ 173.74 ppm (C-1), oxygenated methylene carbon at δ 74.09 ppm (C-1'), other methylene carbon signals between δ 39.18-22.71 ppm and methyl carbons at δ 14.19 (C-27') and 14.13 (C-6) ppm. The HMBC spectrum of **2** showed correlations of H_2-2, H_2-3 and H_2-1' with C-1; H_2-2' and H_2-3' with C-1'; H_2-5 with C-6; and H_2-26' with C-27'. On the basis of these evidences the structure of **2** has been elucidated as *n*-heptacosanyl *n*-hexanoate, a new fatty ester (Fig. 1).

Compound **3**, designated as β-sitosterol diglucoside, was obtained as a colorless, amorphous powder from chloroform-methanol (9 : 1, v/v) eluants. It gave a positive Liebermann Burchard test for sterols and tests for glycosides. Its IR spectrum exhibited absorption bands for hydroxyl groups (3450, 3404, 3380 cm^{-1}) and unsaturation (1645 cm^{-1}). On the basis of mass and ^{13}C NMR spectra, the molecular ion peak of **3** was determined at *m*/*z* 738 consistent with a molecular formula of steroidal diglucoside $C_{41}H_{70}O_{11}$. The fragment ions arising at *m*/*z* 413 [M-glycone]$^+$, 398 [413-Me]$^+$, 273 [413-$C_{10}H_{21}$, side chain]$^+$, 255 [273-H_2O]$^+$, 240 [255-Me]$^+$ and 213 [255-ring D fission]$^+$ were characteristic for β-sitosterol aglycone. The ^1H NMR spectrum **3** exhibited three one-proton doublets at δ 5.55 (*J* = 5.1 Hz), 5.32 (*J* = 7.8 Hz) and 4.90 (*J* = 7.7 Hz) ppm ascribable to vinylic H-6 and anomeric H-1' and H-1'' protons, respectively. The other sugar protons appeared between δ 4.48-3.07 ppm. A one-proton broad multiplet at δ 3.54 ppm with half-width of 18.3 Hz was assigned to oxygenated methine H-3α proton. Two three-proton broad singlets at δ 0.99 and 0.65 ppm and four three-proton doublets at δ 0.95 (*J* = 6.1 Hz, Me-21), 0.88 (*J* = 5.8 Hz, Me-27), 0.84 (*J* = 6.0 Hz, Me-26), 0.82 (*J* = 6.1 Hz, Me-29) ppm were associated with tertiary C-19 and C-18, secondary C-21, C-27 and C-26 and primary C-29 methyl protons, all attached to saturated carbons. The ^{13}C NMR spectrum of **3** displayed signals for 41 carbons. The important signals appeared for vinylic carbons (δ 140.45, C-5; 121.18, C-6 ppm), anomeric carbons (δ 103.48 ppm, C-1'; 100.81 ppm, C-1''), oxygenated methine carbon (δ 73.46 ppm, C-3) and hydroxymethylene carbons (δ 61.09 ppm, C-6'; 60.08 ppm, C-6''). The presence of H-4' signal as a multiplet at δ 3.50 ppm in the ^1H NMR spectrum and ^{13}C NMR signal in the deshielded region at δ 68.32 ppm (C- 4') suggested (4'→1'') linkage of the sugar units. The ^1H NMR and ^{13}C NMR spectral data of the steroidal nucleus were compared with other stigmastene-type molecules (16-18). The ^1H-^1H COSY spectrum of **3** showed correlations of H_2-1, H_2-2 and H_2-4 with H-3; H_2-4, H-8 and H_2-7 with H-6; H-3, H-2' and H-5' with H-1'; and H-4' and H-2'' with H-1''. The HMBC spectrum of **3** exhibited interactions of H_2-1, H_2-2 and H_2-4 with C-3; H_2-4, H-6, H_2-7 with C-5; H-3, H-2' and H-5' with C-1'; and H-4', H-2'' and H-5'' with C-1''. Acid hydrolysis of **3** yielded D-glucose and β-sitosterol, co-TLC comparable with the authentic samples. On the basis of these findings the structure of **3** was established as stigmasta-5-en-3-ol-3-*O*-β-D-glucopyranosido-(4'→1'')-*O*-β-D-glucopyranoside (Fig. 1).

Compound **4**, named vesicariabiflavanone A, was obtained as a yellow amorphous powder from chloroform-methanol (19 : 1, v/v) eluants. It gave positive tests of flavonoids and showed UV absorption maxima at 255, 288, 370 nm characteristics of flavanones (19). Its IR spectrum displayed absorption bands for hydroxyl groups (3410, 3380, 3310 cm^{-1}), carbonyl group (1705 cm^{-1}) and unsaturation (1640, 1695 cm^{-1}). On the basis of mass and ^{13}C NMR spectra, the molecular ion peak of **4** was determined at *m*/*z* 658 consistent with the molecular formula a biflavonoid $C_{35}H_{30}O_{13}$. The generation of the important fragment peaks at *m*/*z* 356 $[C_{20}H_{20}O_6]^+$ and 302 $[C_{15}H_{10}O_7]^+$ supported biflavonoid nature of the compound. An ion peak at *m*/*z* 152 $[C_8H_8O_3]^+$ arising due to RDA fission further substantiated the presence of a flavanol unit linked to a flavanone of the biflavonoid. The ^1H NMR spectrum of **4** displayed two one-proton singlets at δ 6.94 and 6.83 ppm assigned to aromatic H-6 and H-6a protons, respectively. Three one-proton doublets at δ 6.76 (*J* = 7.8 Hz), 6.72 (*J* = 7.5 Hz), and 6.65 (*J* = 7.5 Hz) ppm were ascribed correspondingly to *ortho*-coupled H-5', H-5'a and H-6' aromatic protons. A one-proton doublet at δ 6.70 (*J* = 2.5 Hz) ppm and a one-proton double doublet at δ 6.68 (*J* = 2.5, 7.8 Hz) ppm were accounted to *meta*-coupled H-2'a and *meta-, ortho*-coupled H-6'a protons, respectively. A one-proton broad multiplet centered at δ 5.25 ppm was attributed to vinylic methine H-3'' proton whereas H_2-4'' vinylic methylene protons appeared as two one-proton broad singlets at δ 5.07 and 5.04 ppm. A one-proton double doublet in the non-aromatic region at δ 4.98 (*J* = 13.1, 2.9 Hz) ppm and a one-proton doublet at δ 4.95 (*J* = 11.1 Hz) ppm were due to the oxygenated methine H-2 and H-2a, respectively. A one-proton doublet at δ 4.48 (*J* = 11.1 Hz) ppm and a three-proton broad singlet at δ 3.31 ppm were accounted to α-oriented H-3a carbinol and methoxy protons, respectively. Two one-proton double doublets at δ 3.11 (*J* = 13.1, 17.2 Hz) ppm and 2.93 (*J* = 2.9, 17.1 Hz) ppm were due to methylene H_2-3*ax* and H_2-3*eq* protons, supporting flavanone ring system of one of the unit of the biflavonoid skeleton (20-22). The methylene protons of the side chain resonated as two-proton multiplets at δ 2.73 (H_2-1'') and 2.61 (H_2-2'') ppm. Its ^{13}C NMR spectrum showed important signals for carbonyl carbons (δ 196.04, C-4; 194.90, C-4a ppm), vinylic carbons of side chain (δ 129.03, C-3''; 102.47, C-4'' ppm), oxygenated methine carbons (δ 82.57, C-2; 80.51, C-2a ppm), hydroxymethine carbon at δ 71.39 (C-3a) ppm and methoxy carbon (δ 55.04 ppm). The substituted aromatic carbons C-8 and C-8a appeared at δ 94.62 and

δ 92.53 ppm, respectively. The ^{1}H-^{1}H COSY spectrum of **4** showed correlations of H-2 with H-3 and H-6'; H-5' with H-6'; H-3'' with H_2-1'', H_2-2'' and H_2-4''; H-2a with H-3a, H-2'a and H-6'a; and H-5'a with H-6'a. In the HMBC spectrum of **4** H-6 interacted with C-5 and C-7; H-2 and H-3 correlated with C-4; H-2, H-5', H-6' interacted with C-1'; H-5' and OMe correlated with C-5'; H_2-1'', H_2-2'' and H_2-4'' interacted with C-3''; H-2a and H-3a interacted with C-4a; and H-2a, H-6'a and H-2'a interacted with C-1'a. On the basis of above discussion, the structure of **4** was elucidated as (2a,3a-*trans*)-3a(β),5a,7a, 3'a,4'a-pentahydroxyflavanolyl-(8a→2')-5,7,3'-trihydroxy-4'-methoxy-8-*n*-but-3''-enyl-flavanone, a new natural biflavonoid (Fig. 1).

Compound **5**, designated vesicariabiflavanone B, was obtained as a yellow amorphous powder from chloroform-methanol (19 : 1, v/v) eluants. Its UV spectrum showed absorption maxima at 242, 291, 366 nm characteristics of flavanones (19). Its IR spectrum displayed absorption bands for hydroxyl groups (3485, 3404, 3380 cm^{-1}), carboxyl group (1695 cm^{-1}), carbonyl function (1685 cm^{-1}) and aromatic ring (1640, 1550, 1065 cm^{-1}). On the basis of its mass and ^{13}C NMR spectra its molecular ion peak was determined at *m*/*z* 766 corresponding to a molecular formula of a biflavanonoid $C_{37}H_{34}O_{18}$. The ion peaks arising at m/z 166 $[C_9H_{10}O_3]^+$ formed due to retro-Diels-Alder fragmentation and at *m*/*z* 317 $[C_{5'} - C_{8a}$ fission, $C_{16}H_{13}O_7]^+$ indicated flavanone nature of the compound possessing a methoxy group in the B ring. The ^{1}H NMR spectrum of **5** displayed three one-proton singlets at δ 6.96, 6.88 and 6.76 ppm assigned to aromatic H-6, H-6a and H-6', two one-proton doublets at δ 6.71 (J = 2,5 Hz) and 6.67 (J = 2.5 Hz) ppm ascribed to *meta*-coupled H-2'a and H-6'a, respectively, two one-proton multiplets at δ 5.76 ($w_{1/2}$ = 8.5 Hz) and 5.36 ($w_{1/2}$ = 8.3 Hz) ppm attributed to *cis*-oriented vinylic H-4'' and H-5'', respectively, two one-proton double doublets at δ 5.28 (J = 12.8, 2.9 Hz), 5.16 (J = 12.6, 2.7 Hz, ppm), and a one-proton doublet at δ 4.51 (J = 6.3 Hz) ppm due to correspondingly oxygenated methine H-2 and H-2a and β-oriented carbinol H-1'', a one-proton multiplet at δ 3.71 ppm with half-width of 14.7 Hz assigned to α-oriented carbinol H-2'' and methoxy protons as a three-proton singlet at δ 3.15 ppm. Four one-proton double doublets at δ 3.12 (J = 17.2, 12.8 Hz), 3.08 (J = 17.3, 12.7 Hz), 2.85 (J = 2.9, 17.2 Hz), 2.80 (J = 2.7, 17.3 Hz) ppm were due to methylene H_2-3 and H_2-3*a* supporting flavanone nature of the molecule (20-22). The remaining methylene of the side chain appeared at δ 2.95, 2.90, 2.64 and 2.48 ppm. Further evidences in support of the structure of compound **5** were drawn from its ^{13}C NMR spectrum which showed important signals for carboxylic carbon (δ 188.02 ppm, C-7''), carbonyl carbons (δ 197.85 ppm, C-4; 196.41 ppm, C-4a), vinylic carbons of side chain (δ 128.22 ppm, C-4''; 127.23 ppm, C-5''), oxygenated methine carbons (δ 78.59 ppm, C-2; 78.20 ppm, C-2a) and methoxy carbon (δ 55.72 ppm). Substituted aromatic carbons C-8, C-8a and C-2' appeared at δ 95.14, 93.33 and 115.51 ppm, respectively. The ^{1}H-^{1}H COSY spectrum of **5** showed correlations of H-2 with H-3; H-1'' with H-2''; H_2-3'', H-4'' and H_2-6 with H-5''; and H-2a with H-3a. The HMBC spectrum of **5** exhibited interactions of H-3 with C-4; H-2 and H-6' with C-1'; H-6 with C-7; H-6a with C-7a; H-3a with C-4a; H-2a, H-2'a and H-6'a with C-1'a. On the basis of above discussion, the structure of **5** was elucidated as 5,7,3',4',5'-pentahydroxy-8-(cis-1''α, 2''β-dihydroxyhept-4''-enyl-7''-oic acid)-flavanoyl-(2'→8a)-5a,7a,3'a,5'a-tetrahydroxy-4'a-methoxyflavanone, a new biflavonoid (Fig. 1).

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HPLC PROFILING OF PHENOLIC ACIDS AND FLAVONOIDS AND EVALUATION OF ANTI-LIPOXYGENASE AND ANTIOXIDANT ACTIVITIES OF AQUATIC VEGETABLE *LIMNOCHARIS FLAVA*

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Abstract: *Limnocharis flava* is an edible wetland plant, whose phenolic acid and flavonoid compositions as well as bioactivities were underexplored. This study analyzed the profiles of selected hydroxybenzoic acids, hydroxycinnamic acids and flavonoids in the aqueous extracts of *L. flava* leaf, rhizome and root by high performance liquid chromatography (HPLC). Anti-lipoxygenase and antioxidant (iron chelating, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, and nitric oxide (NO) scavenging) activities of the extracts were also evaluated. Leaf extract had the highest phenolic contents, being most abundant in *p*-hydroxybenzoic acid (3861.2 nmol/g dry matter), ferulic acid (648.8 nmol/g dry matter), and rutin (4110.7 nmol/g dry matter). Leaf extract exhibited the strongest anti-lipoxygenase (EC_{50} 6.47 mg/mL), iron chelating (EC_{50} 6.65 mg/mL), DPPH scavenging (EC_{50} 15.82 mg/mL) and NO scavenging (EC_{50} 3.80 mg/mL) activities. Leaf extract also had the highest ferric reducing ability. This is the most extensive HPLC profiling of phenolic acids and flavonoids in *L. flava* to date. In conclusion, *L. flava* leaf is a source of health-promoting phenolics, anti-lipoxygenase agents and antioxidants.

Keywords: antioxidant, flavonoid, hydroxybenzoic acid, hydroxycinnamic acid, lipoxygenase inhibition, metal chelating

Worldwide, there is an increasing interest to search for natural antioxidants and other therapeutically relevant bioactive constituents from edible plants. A large number of plant food-derived bioactive compounds reported in the literature are members of the phenolic acid and flavonoid families (1, 2). Many phenolic acids and flavonoids exhibit chemopreventive or therapeutic properties (3, 4). For instance, protocatechuic acid, a hydroxybenzoic acid, exhibits growth inhibitory effects against numerous human cancer cell lines (5-7). Sinapic acid, a hydroxycinnamic acid, shows protective effect against cardiac hypertrophy and dyslipidemia in animal models (8). Rutin, a flavonoid, upregulates activities of antioxidative enzymes such as superoxide dismutase and catalase in cerebral ischemia injury in rats (9). Several studies also revealed that rutin is a potent anti-inflammatory phytochemical (10-12).

Lipoxygenase (LOX) pathway plays an important role in the inflammatory response in the human body (13). The main enzyme involved in the LOX pathway is 5-LOX. The increased activity of this enzyme has been correlated with certain diseases, including asthma (14) and inflammatory bowel diseases (15). Thus, 5-LOX is a potential therapeutic target for such diseases and there is strong interest among researchers to search for natural products with anti-LOX activity. On the other hand, there is also continuing interest among researchers to search for plant food-derived antioxidants, which are perceived to have fewer side effects than synthetic antioxidants commonly in use at present (16).

A number of aquatic plants are cultivated or harvested from the wild and consumed as vegetables (17). Nevertheless, literature substantiating the health-promoting and/or therapeutic potential of such edible aquatic plants is overall limited. Information obtained from investigations on the bioactive constituents and bioactivities of edible aquatic plants may help to promote their utilisation, hence boosting their economic values, as vegetables.

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Moreover, it may promote the application of edible aquatic plants in the development of nutraceuticals or functional food, in addition to their application as an alternative bioresource for the management of human diseases.

Limnocharis flava (L.) Buchenau (Alismataceae), commonly known as yellow velvetleaf, is an edible aquatic plant. *L. flava* inhabits shallow swamps, ditches and stagnant fresh water. The bud, flower, and leaves of the plant are consumed as salad or cooked vegetables in Vietnam, Indonesia, Bangladesh (18), Thailand (19) and Malaysia. At present, there is a gap of knowledge about the health-promoting phenolic constituents and bioactivities of *L. flava*. Thus, this study was undertaken to analyze the profiles of health-promoting phenolic acids and flavonoids in *L. flava*. In addition, the anti-LOX, iron chelating, radical scavenging, and ferric reducing antioxidant activities of this edible aquatic plant were also evaluated.

EXPERIMENTAL

Collection of plant samples and species identification

Healthy specimens of *L. flava* were collected from shallow streams in the town of Tronoh Mines, Perak State, Malaysia. The species of the plant was verified by Professor Hean-Chooi Ong, University of Malaya. Herbarium voucher was stored at the Faculty of Science, Universiti Tunku Abdul Rahman, for future reference.

Preparation of aqueous extracts

The plant samples were rinsed under running tap water to wash off sands and mud. The plant was separated into three parts, namely leaf, rhizome and root, and dried in an oven at 45°C for 48 h. The dried samples were then pulverized to powder by using a blender. Next, the plant powder was suspended in deionized water at a ratio of 1 : 20 (dry weight : volume) and incubated in a 95°C water bath for 2 h with continuous agitation (120 rpm). Subsequently, the suspension was vacuum-filtered using cheesecloth and Buchner funnel. The filtrates were centrifuged at 9000 rpm and 4°C for 10 min. The supernatant was aliquoted and stored at -20°C until further use. The concentration of the supernatant was taken as 50 mg dry matter (DM)/mL.

High performance liquid chromatography (HPLC) analysis

The HPLC system used in this analysis was comprised of Shimadzu LC-20D binary pumps, Shimadzu CTO-10AS column oven, and Shimadzu Prominence SPD-20A UV/Vis detector. Phenomenex-Gemini 5 µm (150 mm length × 4.6 mm internal diameter, 110 Å pore size, 5 µm particle size) was the C-18 reversed phase column used. The choice of solvent, solvent composition and elution program were adopted from (20, 21) with minor modifications. Gradient elution was executed in this analysis with a flow rate of 0.8 mL/min, at an oven temperature of 38°C and injection volume of 20 µL. The mobile phase were deionized water acidified with acetic acid (pH 2.8) as solvent A and acetonitrile as solvent B. Gradient elution was performed as follows: 0-5 min, 5-9% solvent B; 5-15 min, 9% solvent B; 15-22 min, 9-11% solvent B; 22-38 min, 11-18% solvent B; 38-43 min, 18-23% solvent B; 43-44 min 23-90% solvent B; 44-45 min, 90-80%, solvent B; 45-55 min, 80% solvent B; 55-60 min, and 80-5% solvent B. The column was flushed and equilibrated with 5% acetonitrile for 20 min at the completion of each gradient elution program. Phenolic compounds were identified by comparing their respective retention times with those of pure external standards. Hydroxybenzoic acid standards used were protocatechuic acid (PCCA), *p*-hydroxybenzoic acid (*p*-HBA), gallic acid (GA), and vanillic acid (VA). Hydroxycinnamic acid standards used were ferulic acid (FA), caffeic acid (CFA), *p*-coumaric acid (*p*-CA), sinapic acid (SNA), and chlorogenic acid (ChA). Flavonoid standards used were myricetin, rutin and quercetin. Detection wavelengths for hydroxybenzoic acids, hydroxycinnamic acids and flavonoids were 280, 320 and 370 nm, respectively. Chromatograms with positive detection were analyzed using the LabSolution software, compared with standards calibration curves.

Anti-lipoxygenase (LOX) assay

Anti-LOX activity was determined with the microplate-based method reported by (22). First, 20 µL of extract (0-50 mg/mL) was pipetted into a well containing 50 µL of 440 ng/mL LOX prepared in 50 mmol/L Tris-HCl (pH 7.4). The microplate was then incubated in darkness for 5 min at room temperature. Subsequently, 50 µL of 616 µmol/L linoleic acid was added to the mixture, followed by a further incubation of 20 min. Next, 100 µL of freshly prepared ferric oxidation of xylenol orange (FOX) reagent was added to the mixture. The mixture was incubated in the dark again for 30 min. The absorbance of the mixture was determined at 560 nm. FOX reagent was prepared by adding 15 µmol/L xylenol orange and 15 µmol/L $FeSO_4$ into a mixture of 15 mL of 300 mmol/L H_2SO_4 and 135

mL of methanol. Nordihydroguaiaretic acid was used as the positive control. The lipoxygenase inhibition activity was calculated by using the formula:

$$\text{Anti-LOX activity (\%)} = [(A_{control} - A_{sample})/ A_{control})] \times 100$$

where $A_{control}$ is the absorbance of the reaction mixture in which the extract was excluded; A_{sample} is the absorbance of the reaction mixture containing an extract. EC_{50} value is defined as the extract concentration required to achieve 50% inhibition of LOX activity.

Determination of iron chelating activity

The iron chelating assay described in (20) was modified into a microplate format. First, 80 µL of 0.1 mmol/L $FeSO_4$ was pipetted into a well followed by 80 µL of plant extract (0-50 mg/mL). The mixture was incubated for 5 min at room temperature. Next, 160 µL of 0.25 mmol/L ferrozine was added into the mixture, followed by an incubation for 10 min at room temperature. The absorbance of the reaction mixture was measured at 562 nm. Disodium ethylenediaminetetraacetic acid (disodium EDTA) was used as the positive control. Iron chelating activity was calculated by using the formula:

$$\text{Iron chelating activity (\%)} = [(A_{control} - A_{sample})/ A_{control})] \times 100$$

where $A_{control}$ is the absorbance of the reaction mixture in the absence of a plant extract; A_{sample} is the absorbance of the reaction mixture containing a plant extract. EC_{50} value is defined as the extract concentration required to achieve 50% iron chelating activity.

Antioxidant assays

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity

The method described in (23) was modified into a microplate assay. The assay was started by pipetting 10 µL of extract (0-50 mg/mL) to a well, followed by 300 µL of freshly prepared 0.004% (weight/volume) methanolic DPPH solution. The mixture was then incubated in darkness for 30 min at room temperature. The absorbance of the mixture was determined at 517 nm. Ascorbic acid was the positive control used. DPPH scavenging activity was calculated by using the formula:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control})] \times 100$$

where $A_{control}$ is the absorbance of the reaction mixture from which plant extract was omitted; A_{sample} is the absorbance of reaction mixture containing a plant extract. EC_{50} value is defined as the extract concentration required to scavenge 50% of DPPH free radical in the reaction medium.

Determination of nitric oxide (NO) scavenging activity

NO scavenging activity was determined by using the method reported in (24). Firstly, 90 µL of extract (0-50 mg/mL) was pipetted into a well, followed by addition of 30 µL of 5 mmol/L sodium nitroprusside prepared in phosphate buffer saline (pH 7.4). The microplate was then incubated under light at room temperature for 150 min. Next, 90 µL of freshly prepared Griess reagent (1% sulfanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid) was added into the well. The microplate was further incubated in the dark for 10 min after which the absorbance of the reaction mixture was measured at 560 nm. Ascorbic acid was used as the positive control. NO scavenging activity was calculated by using the formula:

$$\text{NO scavenging activity (\%)} = [(A_{control} - A_{sample})/ A_{control})] \times 100$$

where $A_{control}$ is the absorbance of the reaction mixture in which the plant extract was excluded; A_{sample} is the absorbance of reaction mixture containing a plant extract. EC_{50} value is defined as the extract concentration required to scavenge 50% of NO free radical in the reaction medium.

Determination of Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was modified from (25) into a microplate assay. FRAP reagent was prepared freshly by mixing 300 mmol/L acetate buffer pH 3.6, 10 mmol/L 2,4,6-tripyridyl-s-triazine in 40 mmol/L HCl and 20 mmol/L $FeCl_3 \times 6H_2O$ at a ratio of 10 : 1 : 1. A standard calibration curve was constructed with $FeSO_4 \times 7H_2O$ (0.1 to 1.0 mmol/L). The assay was performed by pipetting 10 µL of extract into a well, followed by 300 µL of FRAP reagent. The microplate was then incubated for 5 min at room temperature. The absorbance of the reaction mixture was measured at 593 nm. FRAP values were expressed as µmol of Fe^{2+} equivalents per g of DM of plant sample. Butylated hydroxytoluene (BHT) was used as the positive control.

Data analysis

Experiments were performed in triplicates. Results were expressed as the mean ± standard errors. Statistical Analysis System (SAS) software version 9.2 was used for statistical analysis. Data collected were analyzed by using the one-way ANOVA test. Fisher's Least Significant Difference (LSD) test was used to separate means of significant differences at $\alpha = 0.05$. The determination of EC_{50} values for bioactivities was carried out by using lin-

ear regression analysis performed with Microsoft Office Excel 2007.

RESULTS

Hydroxybenzoic acid, hydroxycinnamic acid and flavonoid contents

Leaf and rhizome extracts contained all four types of hydroxybenzoic acids, namely PCCA, *p*-HBA, GA and VA (Table 1). The highest *p*-HBA content was detected in the leaf extract, which accounts for approximately 0.05% of plant DM by weight. PCCA contents were similar between leaf and rhizome extracts, which were about 1.6-fold higher compared with root extract. GA and VA were present in all extracts but both were the most abundant in the leaf extract.

Among the six hydroxycinnamic acids analyzed, SA was not detected in any of the extracts (data not shown). Leaf extract also had the highest CFA, SNA and ChA contents (Table 1). FA was only found in leaf extract, which accounts for 0.012% of plant DM by weight.

The abundance of myricetin, rutin and quercetin in the plant extracts is presented in Table 1. Myricetin was present in all three extracts. Leaf extract had the highest myricetin content, which was 8.8-fold and 3.2-fold higher compared with the rhizome and root extracts, respectively. Rutin was detected only in the leaf extract at about 0.25% of plant DM by weight. Leaf extract also had the highest quercetin content, which was 5.6-fold greater than that in the root extract.

Anti-LOX and antioxidant activities

Only leaf and root extracts exhibited anti-LOX activity within the range of extract concentration tested. Anti-LOX activities of the leaf and root extracts increased in an extract concentration-dependent manner at 0-10 mg/mL and 0-50 mg/mL, respectively (data not shown). The EC_{50} of leaf extract was 4.5-fold lower than that of root extract (Table 2). The EC_{50} values of both of the extracts were higher compared with nordihydroguaiaretic acid.

All three extracts had iron chelating activity, showing concentration-dependent increases at 0-10 mg/mL (data not shown). The EC_{50} value of the leaf extract was approximately 64% lower than those of rhizome and root extracts (Table 2). The EC_{50} values of rhizome and root extracts were similar. All the extracts had significantly higher EC_{50} values compared with disodium EDTA.

DPPH radical scavenging activity in all three extracts increased in an extract concentration-dependent manner at 0-50 mg/mL (data not shown). The EC_{50} values of the rhizome and root extracts were about 9-fold higher than that of the leaf extract (Table 2). The EC_{50} value of the leaf extract was 51-fold greater compared with ascorbic acid.

All three extracts of *L. flava* also showed NO radical scavenging activity, which was observed to be concentration-dependent at 0-10 mg/mL (data not shown). Leaf extract had the lowest EC_{50} value, which was 78% lower than those of rhizome and root extracts (Table 2). The EC_{50} value of leaf extract was 25-fold higher compared with ascorbic acid. The rhizome and root extracts had similar EC_{50} values.

Table 1. Hydroxybenzoic acid, hydroxycinnamic acid, and flavonoid contents of *L. flava* extracts.

Extracts*		Leaf	Rhizome	Root
Hydroxybenzoic acids (nmol/g DM)	PCCA	1298.4 ± 7.3 [a]	1327.7 ± 20.7 [a]	805.3 ± 25.2 [b]
	p-HBA	3861.2 ± 64.9 [a]	83.1 ± 2.0 [b]	N.D.
	GA	234.0 ± 7.8 [a]	117.7 ± 3.6 [b]	66.1 ± 3.3 [c]
	VA	206.0 ± 4.8 [a]	65.8 ± 1.4 [b]	71.1 ± 1.8 [b]
Hydroxycinnamic acids (nmol/g DM)	FA	648.8 ± 9.5 [a]	N.D.	N.D.
	CFA	332.0 ± 1.6 [a]	N.D.	155.0 ± 3.7 [b]
	p-CA	61.5 ± 0.7 [a]	N.D.	103.3 ± 2.0 [b]
	SNA	86.6 ± 1.9 [a]	4.3 ± 0.1 [b]	36.8 ± 0.2 [c]
	ChA	54.8 ± 4.2 [a]	15.8 ± 0.4 [b]	27.6 ± 1.1 [c]
Flavonoids (nmol/g DM)	Myricetin	631.2 ± 15.4 [a]	71.8 ± 1.1 [b]	196.6 ± 5.8 [c]
	Rutin	4110.7 ± 67.1 [a]	N.D.	N.D.
	Quercetin	25.4 ± 0.7 [a]	N.D.	4.5 ± 0.1[b]

Data are presented as the mean ± standard errors (n = 3). Values in the same row that are followed by different superscript letters ([a-c]) are significantly different ($p < 0.05$), as determined by Fisher's LSD test. * Abbreviations - see text. N.D. = not detectable.

Table 2. EC_{50} values for bioactivities of *L. flava* extracts.

Extracts	EC_{50} values (mg/mL)			
	Anti-LOX activity	Iron chelating activity	DPPH scavenging activity	NO scavenging activity
Leaf	6.47 ± 0.05 [a]	6.65 ± 0.04 [a]	15.82 ± 0.21 [a]	3.80 ± 0.02 [a]
Rhizome	N.D.	24.88 ± 0.17 [b]	137.98 ± 1.03 [b]	16.89 ± 0.07 [b]
Root	29.14 ± 0.02 [b]	22.92 ± 0.99 [b]	148.59 ± 0.41 [c]	17.98 ± 0.17 [c]
Positive control	0.12 ± 0.00 [c] (Nordihydroguaiaretic acid)	0.02 ± 0.00 [c] (Disodium EDTA)	0.31 ± 0.01 [d] (Ascorbic acid)	0.15 ± 0.00 [d] (Ascorbic acid)

Data are presented as the mean ± standard errors (n = 3). In each column, values followed by different superscript letters ([a-d]) are significantly different ($p < 0.05$), as determined by Fisher's LSD test. N.D. = not detectable.

Among the three extracts, leaf extract had the highest FRAP value (79.9 µmol/g), followed by rhizome extract (12.3 µmol/g), and root extract (10.4 µmol/g). The FRAP value of the leaf extract was approximately half of that of BHT (173.3 µmol/g).

DISCUSSION

HPLC analysis of the leaf, rhizome and root extracts of *L. flava* revealed different phytochemical compositions. Leaf extract was the most abundant source of three hydroxybenzoic acids (*p*-HBA, GA, and VA), four hydroxycinnamic acids (FA, CFA, SNA, ChA) and three flavonoids (myricetin, rutin and quercetin). The results point to the leaves being the richest source of health-promoting phenolic acids and flavonoids in the *L. flava* plant. This highlights the potential health benefits of *L. flava* leaves when consumed as vegetable.

The flavonoid profile of *L. flava* plant has only been investigated in one previous study (26), whereas phenolic acid composition of the plant has not been reported in the literature. Hence, this study is the most comprehensive profiling of phenolic constituents of *L. flava* to date, encompassing not only flavonoids, but also hydroxybenzoic and hydroxycinnamic acids. Yang et al. (26) did not detect any myricetin and quercetin in *L. flava* leaf extract. In this study, these two flavonoids occurred in the leaf and root extracts. Such discrepancy may have arisen from the different extraction strategies used in their and this studies. In this study, we have extracted *L. flava* with hot water. In comparison with organic solvent extraction, hot water extraction should resemble more closely the way *L. flava* leaves are likely to be prepared for human consumption. Thus, the HPLC profiling of phenolic acids and flavonoids is likely to give a relatively realistic representation of the phytochemical contents available in *L. flava* when it is consumed as cooked vegetable.

The contents of some phenolic constituents in the *L. flava* leaf extract were higher compared with some popularly consumed vegetables and food plants. For example, *p*-HBA content of *L. flava* leaf extract (3861.2 nmol/g DM, or 533.3 µg/g DM), by itself, was higher than the total contents of hydroxybenzoic acids in green lettuce (264.7 µg/g dry weight), swiss chard (68.9 µg/g dry weight), spinach (219.9 µg/g dry weight) and pea shoots (409.8 µg/g dry weight) (27). On the other hand, CFA content of *L. flava* leaf extract (332.0 nmol/g DM, or 59.8 µg/g DM) was higher than that of green lettuce (9.3 µg/g dry weight) (27). In this study, we report for the first time the detection of rutin and its high abundance in the leaf extract of *L. flava*. The rutin content of *L. flava* leaf extract (4110.7 nmol/g DM, or 2509.7 µg/g DM) surpassed that of raw buckwheat groats (230.1 µg/g dry weight) (28).

To the best of our knowledge, this is the first report of anti-LOX activity in *L. flava*. In this study, hot water extracts of *L. flava* exhibited anti-LOX activity. This suggests that *L. flava*, especially its leaves, is a potential source of water-soluble and thermal-stable LOX inhibitor. Several phenolic acids and flavonoids, such as protocatechuic acid (29), quercetin (30), caffeic acid and *p*-coumaric acid (31), have been reported to exhibit anti-LOX activity. Quercetin also acts synergistically with other active ingredients in spices to inhibit human 5-lipoxidase (30). Thus the higher anti-LOX activity of the *L .flava* leaf extract may be attributed to its higher levels of PCCA, CFA, *p*-CA and quercetin, when compared to rhizome and root extract. Suppression of LOX activity is associated with mitigation of inflammation-related diseases (22). Our finding therefore implies that *L. flava* may be exploited as a novel source of functional food ingredients for the prevention and/or management of

inflammation-related disorders. In addition, *L. flava* leaf extract may serve as a source of vegetable-derived LOX inhibitors which can be developed further as therapeutic agents. High level of NO produced during the course of inflammation can lead to tissue injuries, which are responsible for diseases such as rheumatoid arthritis, asthma, atherosclerosis, autoimmune disease, Alzheimer's disease and diabetes (32). Scavenging of NO is an option as treatment to attenuate the severity of such diseases (33, 34). Hence, *L. flava* leaf extract, with both its NO scavenging and anti-LOX activities may offer greater protective effects against inflammation-related diseases when compared with other natural products possessing only one of these bioactivities.

Leaf extract had the highest antioxidant activities compared with extracts of other parts of the *L. flava* plant. Besides NO scavenging activity, the iron chelating, DPPH scavenging and ferric reducing activities were all at the highest levels in the leaf extract. Such a trend was also observed in other vegetables and edible plants, such as the indigo plant (*Polygonum tinctorium Lour.*) (35), castor plant (*Ricinus communis* L.) (36), and *Acmella oleraceae* Murr. (37). In this study, the iron chelating activity of *L. flava* leaf extract is reported for the first time. Iron chelating activity of vegetables may have certain significance to human health. Iron is vital to the human body and its functions involve oxygen delivery to tissues, gene regulation, and electron transfer reaction (38). Iron deficiency may result in development of diseases such as anemia, glossitis and blue sclera (39). However, iron overload may also lead to iron accumulation and resulting toxicity in the body. Iron may catalyze the formation of reactive oxygen species (ROS), which subsequently can have detrimental effects on the cardiovascular and neurological systems, leading to atherosclerosis, Parkinson's and Alzheimer's diseases (40). Desferriferrioxamine-B is clinically used to keep the level of iron in the body in control, but it will compromise the renal function (41). The discovery of vegetable sources exhibiting iron chelating activity, such as the *L. flava* leaf extract, could contribute to future development of functional food and/or isolation of natural metal chelators to be used for the control of body iron status, potentially with minimal or no side effects.

DPPH radical scavenging activity has been reported for the ethanolic leaf extract (42), ethanolic extract of bud and flower (19), and 70% ethanol extract of the leaves of *L. flava* (43). Hence, our observation agrees with previous finding of the ability of *L. flava* to scavenge DPPH radicals. The FRAP assay is commonly used to evaluate the antioxidant potential of food samples (44). The FRAP value of *L. flava* leaf extract obtained in this study is comparable to those reported for 70% ethanol extract of *L. flava* leaves (43) and 70% ethanol extract of *L. flava* stem (45). In this study, DPPH and NO radical scavenging assays as well the FRAP assay were employed to demonstrate the antioxidant activity of *L. flava* extracts. Natural products may exert their antioxidant activity by more than one mechanism. Thus multiple antioxidant tests were performed in this study as was previously recommended (46). Correlation analysis was not carried out between phytochemical contents and the bioactivity parameters in this analysis due to the small number of extracts analyzed. Nevertheless, to some extent, the trends of relative abundance of VA, GA, SNA, ChA and myricetin in the extracts appear to correspond with the trends of their relative levels of anti-LOX, iron chelating, radical scavenging and ferric reducing activities. Previous reports of antioxidant properties of VA and ChA (47) as well as myricetin (48) lend further support to our proposal that the antioxidant activities detected in *L. flava* can be accounted for at least partially by their hydroxybenzoic acids, hydroxycinnamic acids and flavonoids. In any case, we cannot rule out the possibility that phytoconstituents not analyzed in this study could also have contributed to the detected bioactivities.

CONCLUSION

In conclusion, this study demonstrated that *L. flava* is vegetable that is rich in health-promoting phenolics, with anti-LOX, iron chelating and radical scavenging activities. HPLC analysis revealed the presence of PCCA, GA, VA, SNA, ChA and myricetin in all *L. flava* extracts tested. Overall, leaf extract of *L. flava* was the most abundant source of health-promoting phenolics and exhibited the strongest anti-LOX, iron chelating and radical scavenging activities when compared to rhizome and root extracts.

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EFFECT OF *GYMNEMA SYLVESTRE*, *CITRULLUS COLOCYNTHIS* AND *ARTEMISIA ABSINTHIUM* ON BLOOD GLUCOSE AND LIPID PROFILE IN DIABETIC HUMAN

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Abstract: The aim of this study was to manage diabetes with medicinal plants (*Gymnema sylvestre*, *Artemisia absinthium* and *Citrullus colocynthis*) in human patients with type II diabetes. Thirty two patients of type II diabetes from both sexes of 30-60 years age were registered for this study and distributed them into four groups, each having 8 patients. Capsules of each, *Gymnema sylvestre*, *Artemisia absinthium* and *Citrullus colocynthis* were given to patients twice a day for 30 days in 1 g per day dosage and investigated for glucose, triglyceride (TGL) and cholesterol level. *Gymnema sylvestre* reduced 37% glucose, 5% TGL, 13% cholesterol and 19% low desity lipoproteins (LDL) level in diabetic individuals. *Citrullus colocynth* reduced glucose, cholesterol and TGL and HDL-cholesterol level by 35, 6, 6, and 5%, respectively. *Artemisia absinthium* reduced 3% high desity lipoproteins (HDL) and 6% LDL level. From results, it can be concluded that the powdered *Gymnema sylvestre*, *Citrullus colocynthis*, and *Artemisia absinthium* possess good anti-diabetic features, however these herbal products had no significant effect on lipid profiles of the diabetic human.

Keywords: *Gymnema sylvestre*, *Artemisia absinthium*, *Citrullus colocynthis*, blood glucose, lipid profile, diabetic individuals

Diabetes mellitus is a clinical syndrome characterized by relative or absolute deficiency of insulin or by resistance to the action of insulin at the cellular level (1). It is a disorder of glucose metabolism occurring from dysfunction of pancreatic β cells and insulin resistance, it is characterized by partial or total lack of functioning insulin and there is alteration in carbohydrate, fat and protein metabolism (2).

To manage diabetes with medicinal plants along with dietary restriction has caught the attention of most researchers (3). Even today, natural sources form the basis for a large number of modern drugs and one or more than one active ingredient from them is to be found in 25 percent of all prescriptions (4). There is a dire need for other strategies to the current modern pharmacotherapy of diabetes mellitus. Herbal drugs comprise a significant amount of conventional medicine and literature, which is an indication of anti-diabetic activity exhibited by more than 400 plant species (5).

Gymnema sylvestre (Asclepiadaceae), *Citrullus colocynthis* (Cucurbitaceae) and *Artemisia absinthium* (Asteraceae) have been used for its antidiabetic, antihelmintic, stomachic, antibacterial, antifeedant, antifertility, antipyretic, cytostatic, antitumor, and antimalarial action (6, 7). The literature study has shown the antidiabetic effect of these plants in study subjects with hyperglycemia induced by streptozocin or other compounds. There was no study about antidiabetic effect of these herbal drugs in diabetic patients. Therefore, this study was designed to

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assess the effect of *Gymnema sylvestre*, *Citrullus colocynthis* and *Artemisia absinthium* on blood glucose and lipid profile in type II diabetic individuals of Tehsil Sarai Naurang of District Lakki Marwat, Khyber Pakhtoonkhah (KPK) province, Pakistan.

METHODOLOGY

Location of study

This study was designed and directed by Dongzhimen Hospital, Beijing University of Chinese Medicine, and got supports from other research institutions, and conducted in Tehsil Sarai Naurang of District Lakki Marwat, on type II diabetic individuals, who were properly registered for the study.

Selection criteria, sample size and study design

This study was approved (Approval no. 34-2012/HU/PHM) by the Departmental Review Committee (University of Hazara) and was conducted in accordance with international guidelines for Good Clinical Practice and Hilsinki guidelines for human use in laboratory (8, 9).

The study was advertised through personal contacts, telephone calls to diabetic individuals and by visiting diabetic centers and surrounding villages. The questionnaire contained querries about demography, diabetic type, medication for diabetes and medication for other diseases. Their fasting blood glucose was determined. Those diabetic individuals whose fasting blood sugar level was 125 mg/dL or above, and who were not taking insulin or any other medicines for any other diseases were registered for the study. Thirty-two patients of type II diabetes from both sexes of 30-60 years age (mean age 49.5 years) were registered for the study. These individuals were divided into four groups namely group 1, 2, 3 and 4. Each group was having 8 individuals: Group 1, assigned for *Gymnema sylvestre*, group 2 for *Artemisia absinthium*, group 3 for *Citrullus colocynth* and group 4 for placebo.

Preparation of *Gymnema sylvestre*, *Citrullus colocynthis* and *Artemisia absinthium* capsules

The required amount of *Gymnema sylvestre*, *Citrullus colocynthis* and *Artemisia absinthium* leaf were purchased from the local market and ground finely. The ground herbs were packed into the hard gelatin capsules so that each capsule contained 0.5 g. The capsules were stored in a dry, cool place.

Experimental protocol

The capsules of *Gymnema sylvestre*, *Citrullus colocynthis*, and *Artemisia absinthium* were given 1 g/day dosage (in two divided doses 12 hourly) for 30 days to group 1, group 2 and group 3, respectively. From day 30 to day 40 (10 days) was the wash period and no dose was given in those 10 days; however, blood samples were collected on day 40 to assess the effect of these herbal drugs. One gram placebo doses/day were given for 30 days to group 4.

Blood collection and analysis

Approximately, 5 mL fasting blood samples were taken from each individual of each group on day 0, 10, 20, 30 and 40. Blood samples were transferred to sterilized centrifuge tubes and allowed for clotting at room temperature. The samples were centrifuged for 5 min in a centrifuge (Hiki, China) at 4000 rpm for serum separation. Separated serums were transferred to Eppendorf tubes and were stored in freezer at 0^{o}C for later analysis. Glucose was determined by the method of Barham et al. using Spectrophotometer (S-200D, England) and Randox Kit (CAT No. GL2586/s) (10). Trigliceride was determined by the method of Tietz (11) using Spectrophotometer (S-200D, England) and Randox Kit (REF TR210). Cholesterol was determined by enzymatic colorimetric method as mentioned in the report of the National Cholesterol Education Program (12). Spectrophotometer (S-200D, England) and Randox kit (CAT No. CH 207) were used for this purpose. Low-density lipoproteins (LDL) were precipitated by adding phosphotungstic

Table 1. Information of diabetic individuals on the day of screening.

Group	Age	BMI	Glucose (mg/dL)	TGL (mg/dL)
1	48 ± 7	29.1 ± 7.1	178 ± 35	231 ± 15
2	51 ± 9	28.9 ± 4.2	170 ± 30	219 ± 42
3	49 ± 7	26.9 ± 4.1	169 ± 25	230 ± 20
4	50 ± 9	27 ± 6.2	162 ± 22	210 ± 25

acid and magnesium ions to the sample. Centrifugation left only the HDL (high-density lipoproteins) in the supernatant; their cholesterol content was determined (13, 14).

Statistical analysis

The data were statistically analyzed by analysis of variance and LSD test with $p < 0.05$ using statistical software Mstat C. (The MSTAT C with MGRAPH by Russell D. Freed MSTAT Director, Crop and Soil Science Department, Michigan State University version).

RESULTS AND DISCUSSION

This study was conducted on type II diabetic individuals for 40 days. These individuals were on hypoglycemic drugs but were not on insulin therapy. Their serum glucose concentration, as noted from their previous laboratory reports were either 125 mg/dL or above. The information about these 32 diabetic individuals on the day of screening in terms of age, BMI, serum glucose and serum trigliceride are given in Table 1.

The effect of *Gymnema sylvestre* on the serum glucose and lipid profile is portrayed in Table 2. The values on day 0 indicate the fasting values of the studied biochemical parameters of diabetic individuals before the start of treatment and were considered as control values. On the starting day of experiment (day 0), mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, were 219 ± 41, 218 ± 70l, 274 ± 70, 37 ± 6 and 191 ± 63 mg/dL, respectively. When the diabetic individuals of these groups used 1 g of *Gymnema sylvestre* doses/day for 30 days, their mean fasting serum glucose, TGL, cholesterol, HDL and LDL level changed to138 ± 17, 208 ± 72, 238 ± 56, 43 ± 11 and 154 ± 54 mg/dL, respectively. Use of *Gymnema sylvestre* capsules for 30 days significantly ($p < 0.05$) reduced the serum glucose level of diabetic individuals as compared to serum glucose values on day 0 of this group. The data show that 1 g dose of *Gymnema sylvestre* reduced 37% glucose, 5% TGL, 13% cholesterol, 2% HDL, and 19% LDL level in diabetic individuals. The decrease in glucose level was significant ($p < 0.05$), while the decrease in all other parameters was non-significant ($p > 0.05$). The mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, on day 40 (when they did not receive *Gymnema sylvestre* for the last 10 days) returned back to the control values and their difference from control values was non-significantly different ($p > 0.05$).

The effect of *Citrullus colocynthis* on the serum glucose and lipid profile is given in Table 2. The values on day 0 indicate the fasting values of the studied biochemical parameters of diabetic individuals before the start of treatment and were con-

Table 2. Effect of the studied herbs on various biochemical parameters in diabetic individuals (n = 8, the mean ± SD).

Group	Biochemical parameters (mg/dL)	Values				
		Day 0	Day 10	Day 20	Day 30	Day 40
Gymnema Sylvestre	Fasting glucose	219 ± 41	159 ± 26	157 ± 24	138 ± 17	181 ± 38
	TGL	218 ± 70	218 ± 75	212 ± 74	208 ± 72	230 ± 87
	Cholesterol	274 ± 70	263 ± 62	261 ± 65	238 ± 56	249 ± 54
	HDL	37 ± 6	39 ± 8	41 ± 11	43 ± 11	37 ± 7
	LDL	191 ± 63	180 ± 57	177 ± 61	154 ± 54	166 ± 51
Citrullus colocynthis	Fasting glucose	215 ± 56	188 ± 49	156 ± 48	140 ± 35	213 ± 69
	TGL	253 ± 76	248 ± 74	242 ± 72	238 ± 69	256 ± 74
	Cholesterol	257 ± 80	249 ± 76	249 ± 71	241 ± 72	253 ± 79
	HDL	38 ± 12	39 ± 11	41 ± 10	40 ± 12	38 ± 11
	LDL	168 ± 83	160 ± 79	158 ± 75	153 ± 77	167 ± 77
Artemisia absinthium	Fasting glucose	211 ± 57	204 ± 262	182 ± 43	143 ± 30	191 ± 26
	TGL	187 ± 65	185 ± 64	181 ± 59	169 ± 64	190 ± 68
	Cholesterol	239 ± 35	237 ± 36	234 ± 34	226 ± 32	238 ± 31
	HDL	39 ± 8	39 ± 8	40 ± 9	40 ± 9	39 ± 8
	LDL	163 ± 36	161 ± 36	157 ± 33	153 ± 32	162 ± 38

sidered as control values. On the starting day of experiment (day 0), mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, were 215 ± 56, 253 ± 76l, 257 ± 80, 38 ± 12 and 168 ± 83 mg/dL, respectively. When the diabetic individuals of these groups used 1 g of *Citrullus colocynth* doses/day for 30 days, their mean fasting mean fasting serum glucose, TGL, cholesterol, HDL and LDL level changed to 140 ± 35, 238 ± 69, 241 ± 72, 40 ± 12 and 153 ± 77 mg/dL, respectively. Use of *Citrullus colocynth* capsules for 30 days significantly ($p < 0.05$) reduced the serum glucose level of diabetic individuals as compared to serum glucose values on day 0 of this group. The data show that 1 g dose of *Citrullus colocynthis* reduced 35% glucose, 6% TGL, 6% cholesterol, 5% HDL, and 9% LDL level in diabetic individuals. The decrease in glucose level was significant ($p < 0.05$), while the decrease in all other parameters was non-significant ($p > 0.05$). The mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, on day 40 (when they did not receive *Citrullus colocynthis* for the last 10 days) returned back to the control values and their difference fron control values was non-significantly different ($p > 0.05$).

The effect of *Artemisia absinthium* on the serum glucose and lipid profile is presented in Table 2. The values on day 0 indicate the fasting values of the studied biochemical parameters of diabetic individuals before the start of treatment and were considered as control values. On the starting day of experiment (day 0), mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, were 211 ± 57, 187 ± 65, 239 ± 35, 39 ± 8 and 163 ± 36 mg/dL, respectively. When the diabetic individuals of these groups used 1 g of *Artemisia absinthium* doses/day for 30 days, their mean fasting mean fasting serum glucose, TGL, cholesterol, HDL and LDL level changed to 143 ± 30, 169 ± 64, 226 ± 32, 39 ± 8 and 162 ± 38 mg/dL, respectively. Use of *Artemisia absinthium* capsules for 30 days significantly ($p < 0.05$) reduced the serum glucose level of diabetic individuals as compared to serum glucose values on day 0 of this group. The data show that 1 g dose of *Artemisia absinthium* reduced 32% glucose, 10% TGL, 5% cholesterol, 3% HDL and 6% LDL level in diabetic individuals. The decrease in glucose level was significant ($p < 0.05$), while the decrease in all other parameters was non-significant ($p > 0.05$). The mean fasting serum glucose, TGL, cholesterol, HDL and LDL concentration of the diabetic individuals of group 1, on day 40 (when they did not receive *Artemisia absinthium* for the last 10 days) returned back to the control values and their difference from control values was non-significantly different ($p > 0.05$).

Gymnema sylvestre, *Citrullus colocynthis*, and *Artemisia absinthium* were observed to produce hypoglycemic effect in diabetic human. The hypoglycemic action of these three herbs is already reported in the literature. In those studies, the investigators employed extracts of these herbs in hyperglycemic animal models under the effect of glucose lowering compounds e.g., streptozocin and alloxan (15).

In a study, the authors claimed hypoglycemic effect of alcoholic extract of *Gymnema sylvestre*. Another study also proposed the antihyperglycemic effect in diabetic subjects after oral administration (400 mg per day) of leaf extract. Moreover, other investigators observed the stimulation of insulin secretion from isolated human β-cell and mouse cells *in vitro*, without compromising cell viability after treating with the aqueous extract of *Gymnema sylvestre* leaves (16). Moreover, the ethanolic extract of *Citrullus colocynthis* pulp in an oral dose of 300 mg/kg was observed to significantly diminish plasma glucose concentrations in alloxan-induced diabetic rats. In another study, the aqueous, crude, and purified extracts of *Citrullus colocynthis* pulp also illustrated a dose-dependent hypoglycemic effect through the enhancement of insulin release from isolated islets (16). Various parts of *Citrullus colocynthis* such as roots, fruits, seeds, rinds and leaves, have been used in extract preparation. The doses of these extracts range between 10-500 mg per kg body weight of animals per day (17). In a study, hypoglycemic effect was observed in streptozocin-induced hyperglycemic rats after oral administration of different doses (100, 250 and 500 mg/kg of rat body weight) of *Artemisia absinthium* for six weeks (16).

In addition, all these three herbs, *Gymnema sylvestre*, *Citrullus colocynthis*, and *Artemisia absinthium* produce hypoglycemic effect in a dose dependent manner. Mechanistically, it has been proposed that all the three herbs exert antidiabetic effect through insulinomimetic (to increase insulin secretion) and insulinotropic action. Actually, these herbs are believed to be involved in the regeneration and repairmen of pancreatic β-cells (16).

CONCLUSION

From the results, it can be concluded that the powdered *Gymnema sylvestre*, *Citrullus colocynthis*,

and *Artemisia absinthium* possess good anti-diabetic features, however these herbal products had no significant effect on lipid profiles of the diabetic human.

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PHARMACEUTICAL TECHNOLOGY

EVALUATION OF DICLOFENAC SODIUM SUSTAINED RELEASE MATRIX PELLETS: IMPACT OF POLYETHYLENE GLYCOLS MOLECULAR WEIGHT

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Abstract: Sustained release matrix pellets loaded with 5% w/w diclofenac sodium (DS) were prepared using extrusion/spheronization technique. Different polyethylene glycols (PEGs) of different molecular weight, namely PEG 2000, PEG 4000 and PEG 6000 were mixed with avicel PH 101® in different weight ratios to manufacture the pellet formulations and water was used as a binder. Mix torque rheomter was used to characterize the pellets' wet mass. Also, the prepared pellets were characterized for their particle sizes, DS content, shape and morphology as well as the *in vitro* drug release. The results showed that increasing PEG weight ratio resulted in a reduction of wet mass torque as well as binder ratio, especially at PEG high weight ratios (30% and 50%) and the extent of lowering wet mass peak torque was inversely proportional to PEG molecular weight. The manufactured pellets exhibited size range of 993 to 1085 μm with small span values. The drug release from pellets was governed by the molecular weight of PEG used, since increasing PEG molecular weight resulted in slowing the drug release rate from pellets, but increasing its level resulted in enhancing release rate. This was attributed to increasing pellet wet mass peak torque by increasing PEG molecular weight and lowering it by increasing PEG level. The prepared pellets showed non-Fickian or anomalous drug release or the coupled diffusion/polymer relaxation.

Keywords: matrix pellets, diclofenac sodium, mix torque rheometry extrusion/spheronization, *in vitro* release.

Multiple-unit dosage forms have gained much attention, with single-unit dosage forms, regarding both therapeutic and formulation benefits. Among the various types of multiple-unit dosage forms, pellets have attracted more attention due to their unique clinical and technical advantages. Pellets or spherical granules are produced by agglomerating fine powders with a binder solution. Pellets are defined as spherical, free-flowing granules with a narrow size distribution, typically varying between 500 and 1500 μm for pharmaceutical applications (1). The interest in pellets as dosage forms (filled into hard gelatin capsules or compressed into disintegrating tablets) has been increasing continuously. Several therapeutic advantages could be achieved using pellets as drug delivery system, over the single-unit regimen, such as less irritation of the gastro-intestinal tract and a lowered risk of side effects due to dose dumping (2). In addition, formulation advantages as the better flow properties, less friable dosage form, narrow particle size distribution, ease of coating and uniform packing can be gained with pellets. It was shown that multi-unit dosage forms have gained considerable popularity over conventional single units for controlled release technology. This is due to the rapid dispersion of pellets in the gastrointestinal tract; they maximize drug absorption, reduce peak plasma fluctuations and minimize potential side effects without lowering drug bioavailability (3). Pellets also reduce variations in gastric emptying rates and overall transit times. Thus, intra and intersubject variability of plasma profiles, which are common with single-unit regimens, are minimized.

Different authors formulated matrix pellets for controlled drug delivery systems techniques, which avoid the use of organic solvents during coating procedures, due to stringent global requirements of

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product safety. Also, by formulating sustained release matrix pellets, time and money could be saved by omitting the coating operation. As the level of understanding regarding the toxic effects of these solvents is increasing, industrial hygiene rules and FDA regulations are being tightened world over, limiting the use of these solvents and exposure of workers to these solvents. Therefore, several reports have been published on alternative techniques such as melt granulation (4), melt extrusion (5, 6), melt dispersion (7), and melt solidification (8) for controlled drug delivery systems. In addition, several attempts have been made to modify drug release from multi-particulate oral dosage forms by incorporating various hydrophobic materials into a basic formulation for pellets (9). Such systems retard the penetration of aqueous fluids into the formulation and hence slow the rate of drug release.

The rheological properties of wet masses can be monitored successfully using a mixer torque rheometer (10, 11) so as to formulate pellets of tailored pharmaceutical characteristics. It was shown that the rheological properties of wet mass could affect the release patterns from pellet formulations. Ibrahim (12) showed that mefenamic acid matrix pellets could be successfully correlated with the wet mass characteristic using mixer rheometry. This will help to obtain a controlled release dosage form capable of lowering the risk of side effects and improving patient convenience as an advantage of pellets as a drug delivery system. Also, Mahrous et al. (13) observed that an inverse relationship exists between indomethacin release from the pellets and the peak torque values of the used polymer mixture.

Diclofenac sodium (DS) is a non-steroidal anti-inflammatory drug (NSAID) and belongs to the group of aryl acetic acid derivatives. It is widely used in treatment of rheumatoid arthritis, osteoarthritis and ankylosing spondylitis (14). Because of its short biological half-life (2 h), it is eliminated from plasma compartments of the body within few hours, so frequent administration is necessary to maintain its therapeutic concentration. Thus, DS is an ideal candidate for sustained release purposes (15, 16). Therefore, the formulation of DS as a sustained release dosage form matrix pellets could be an alternative approach to overcome the potential problems in the gastrointestinal tract, in addition to minimizing dosing frequency (17, 18).

The objectives of the present study were to formulate sustained release matrix pellets loaded with DS using extrusion/spheronization technique as an alternative method to coating technique. Different grades of polyethylene glycols (PEG 2000, PEG 4000 and PEG 6000) were used in combination with avicel. The effect of polyethylene glycol molecular weight on the wet mass peak torque, pellets' shapes and sizes will be characterized using mixer torque rheometry and the *in vitro* release rate of the drug loaded pellets will be assessed as well.

EXPERIMENTAL

Materials

DS was kindly supplied by Al-Jazeera Pharmaceutical Industries (Riyadh, KSA). Polyethylene glycols (PEG 6000, PEG 4000 and PEG 2000) were purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks, U.K.). Microcrystalline cellulose (MCC) (Avicel® PH101) was purchased from Serva Feinbiochemica (Heidelberg, Germany). All other materials and solvents used were of reagent or analytical grade and used without further purification.

Methodology

Characterization of pellets wet masses using a mixer torque rheometer

The mixer torque rheometer used in the present study consists of a 135-mL capacity stainless steel bowl equipped with two mixing blades with rotational speed ranging between 20 and 150 rpm (MTR-3, Caleva, Dorset, England). Depending on the bulk density, a sample of 15–30 g of dry powder material is sufficient to cover the mixer blades. The torque is measured directly on the mixer bowl with the help of a torque arm connected from the main body of the mixer to a calibrated load transducer. The used mixer speed for all the studies was 50 rpm. The data acquisition and analyses were carried out by a personal computer using data acquisition system and software package supplied by the equipment manufacturer.

Powders were mixed in turbula mixer (type S27, Erweka, Apparatebau, Germany) and 15 g sample of this dry blend was utilized in the wet massing studies. Two milliliters of granulating fluid were added in multiply additions over 10 wet massing intervals. Each wet massing interval consisted of a one minute mixing period and a 20-second torque data logging (collection) period with the MTR operating at 50 rpm. Mean torque was monitored during the granulation process.

Manufacture of pellets

Water was used as a granulating liquid in the manufacture of matrix pellets loaded with 5% w/w DS. The water volume required for wet massing was

selected according to the highest torque value measured by the rheometer. The compositions of the studied pellet formulations are shown in Table 1. DS and pellets excipients were mixed in turbula mixer at certain weight and the powder mixture was wetted with water. Next, the resulting wet mass was extruded at a speed of 90 rpm with a screen pore size of 1 mm ∅ (Mini Screw Extruder, Model MSE1014, Caleva, Dorset, England). Spheronization was performed in a spheronizer (Model 120, Caleva, Dorset, England) with a rotating plate of regular cross-hatch geometry, at a speed of 700 rpm, for 5 min. Pellets were then dried on a tray in a hot oven at 50-60°C for 6 h.

Drug content

DS content of the manufactured pellets was determined spectrophotometrically at 285 nm in triplicate. Pellets were crushed in a porcelain mortar and about 20 mg of the crushed pellets were dispersed in 250 mL phosphate buffer (pH 6.8) under sonication for 5 min. The supernatant was filtered through a cellulose nitrate filter with pores of 0.2 µm in diameter (Sartorius, Göttingen, Germany) and measured spectrophotometrically (UV-2800 spectrophotometer, Labomed Inc., USA), then MA content was calculated using a pre constructed calibration curve.

Morphological analysis

The morphological characteristics of particles were observed by scanning electron microscopy (SEM). The samples were sputter-coated with thin gold palladium layer under an argon atmosphere using a gold sputter module in a high-vacuum evaporator. The coated samples were then scanned and photomicrographs were taken with an SEM (Jeol JSM-1600, Tokyo, Japan).

Particle size analysis

The size distribution of the manufactured pellets was investigated using laser light diffraction (Mastersizer Scirocco 2000, Malvern Instruments, Grovewood Road, U.K.). For a typical experiment, about 300 mg of pellets were fed in the sample micro feeder. All samples were analyzed 5 times and average results were taken. The pellets 10th (d(0.1)), 50th (d(0.5)) and 90th (d(0.9)) percentiles were used to characterize the pellets size distribution. The approximate mean diameter was taken as the average of d(0.1), d(0.5), and d(0.9) values.

The span value was employed to characterize the pellet size distribution, since a small span value indicates a narrow particle size distribution. It was calculated from the following formula (19):

$$\text{Span} = \frac{D_{90}-D_{10}}{D_{50}}$$

In vitro dissolution studies

The dissolution measurements were performed using an automated dissolution tester (LOGAN Instrument Corp, Somerset, NJ, USA) coupled to an automated sample collector (SP-100 peristaltic pump, Somerset, NJ, USA). The USP dissolution basket method (apparatus 1) was used. MA loaded pellets equivalent to 20 mg DS were added to the 500 mL of dissolution medium (phosphate buffer, pH 7.4). The temperature was maintained at 37 ± 0.5°C. An accurately weighed amount of the pre-

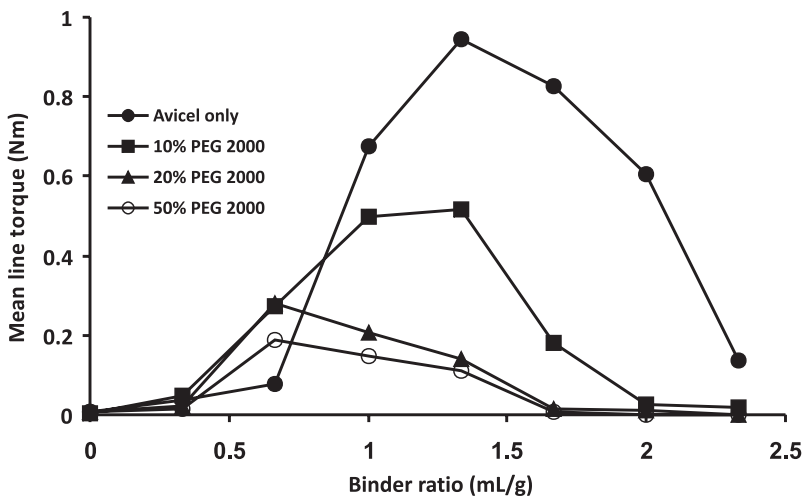

Figure 1. Effect of different concentrations of PEG 2000 on mean torque of Avicel PH101

pared pellets was added to each flask. For each sample formula, drug dissolution was run in triplicate and absorbance was recorded automatically at 285 nm up to 8 h. The percentage of drug dissolved was determined as a function of time.

Statistical analysis

The results were analyzed by using the software GraphPad Prism5 (GraphPad Software, La Jolla, USA) applying one-way ANOVA. Differences between formulations were considered to be significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Wet massing studies

The experiments of wet massing studies were conducted for avicel-PEGs systems in order to establish the water/powder ratio needed to reach a maximum torque response and the effect of PEG grade and level on the pellet wet mass characteristics. Regarding avicel-PEG 2000 systems (Fig. 1) different liquid saturation phases (pendular, funicular and capillary, respectively) were passed through by increasing binder level, with the maximum

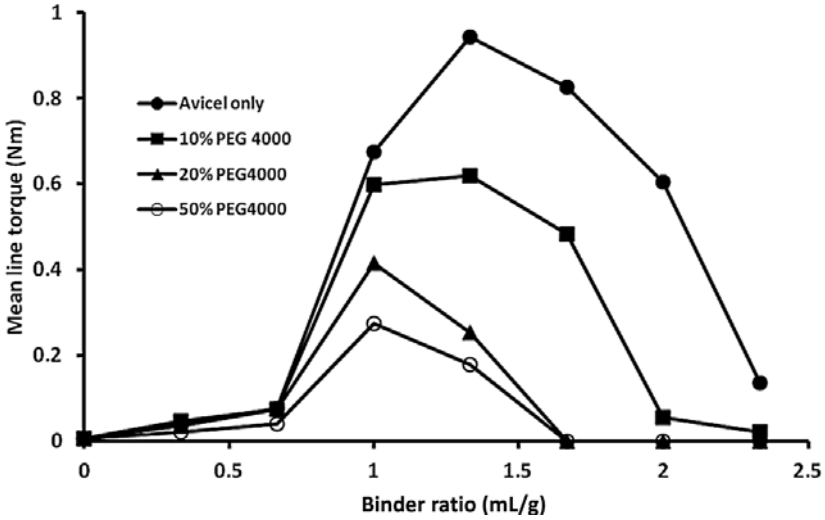

Figure 2. Effect of different concentrations of PEG 4000 on mean torque of Avicel PH101

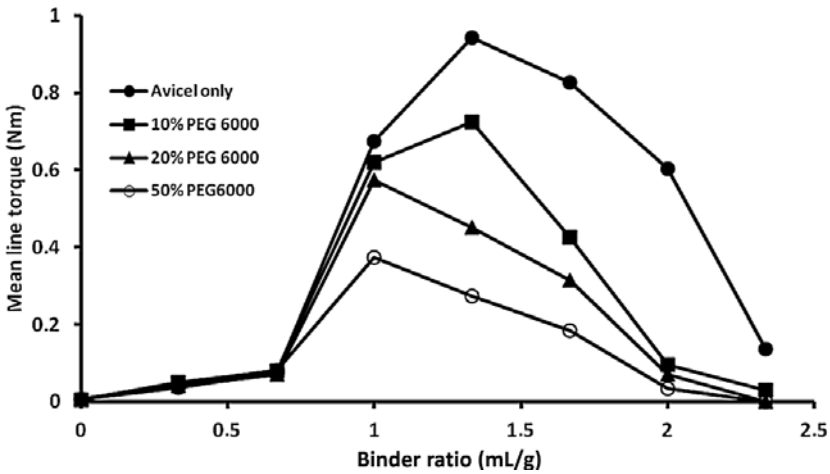

Figure 3. Effect of different concentrations of PEG 6000 on mean torque of Avicel PH101

Table 1. Composition of different pellets formulations loaded with diclofenac sodium.

Formula / Ingredients %	1	2	3	4	5	6	7	8	9	10
Avicel PH 101	85	75	45	43	85	75	45	85	75	45
PEG 2000	10	20	50	50	-	-	-	-	-	-
PEG 4000	-	-	-	2	10	20	50	-	-	-
PEG 6000	-	-	-	-	-	-	-	10	20	50
Diclofenac sodium	5%									
Water (binder)	Q. S.									

torque occurring at the capillary state. Avicel alone exhibited a typical progression of liquid saturation phases. The mean torque value was found to increase with the increase in the wet massing liquid (water) ratio. However, different profiles were detected regarding avicel-PEG 2000 systems, increasing PEG 2000 weight ratio resulted in a severe reduction of the area of MTR curve, i.e., progression of liquid saturation phases occurs at lower water/powder ratio. In addition, reductions of peak torque water/powder ratios (mL/g) and peak torque magnitudes were recorded, which reached the lowest value (0.208 Nm) at 50% w/w PEG 2000 level (Fig. 1). The rheological behaviors of avicel-PEG 4000 systems (Fig. 2) are quite the same as those recorded in case of avicel-PEG 2000. However, there is an increased peak torque in case of avicel-PEG 4000 levels compared to the use of corresponding levels of PEG 2000. For example, upon mixing 50% level of PEG 2000 and PEG 4000 with avicel, the recorded peak torque values were 0.189 Nm and 0.247 Nm, respectively. Similarly, mixing PEG 6000 with avicel for wet massing resulted in increasing the wet mass consistency higher than that measured in case of PEG 2000 or PEG 40000 (Fig. 3). On the other hand, pendular, funicular and capillary phases in case of avicel-PEG 6000 systems were reached at higher peak torque values than those observed in case of the other PEG polymer grades, and the peak torque values were found to decrease by increasing the PEG level. The impact of PEG molecular weight and concentration on the properties of DS pellets wet masses is displayed and summarized in Figure 4. It is clearly evident that high molecular weight grades showed an increase in the mean line torque of the wet mass at all the concentrations studied (10, 20 and 50%) and the mean torque value was found to be decreased by raising polymer level. According to Parker and Rowe (20), the degree of liquid spreading and wetting as well as the substrate binder interaction will determine the relative positions of the peak values of mean line torque. For each polymer concentration, an increase in the mean torque with the increase in the polymer molecular weight at different concentrations resulted in either a sharp or an extended peak followed by a drop in the torque as over-wetting of the powder mass occurred. In addition, the pendular and funicular states are characterized by a progressively increasing network of liquid bridges. Both of these stages will cause an increase in cohesiveness of the powder mass and hence an increased torque on the mixer (21). The capillary state which was reached when all the air spaces in the granular material were filled with liquid occurs at the maximum on the curve. With further addition of liquid the torque decreases as slurry of particles dispersed in liquid is formed.

Drug content

The obtained results showed DS content ranged from 90 to 110% of the theoretical content, which revealed a homogenous drug distribution in the prepared pellets.

Pellets sizes and shapes

The calculated values of volume weighted mean particle size and the d(0.1), d(0.5) and d(0.9) different pellet formulae loaded with DS as determined by laser diffractometry are tabulated in Table 2. One can observe that the volume weighted mean of the manufactured pellets was found to be in the range 993 to 1085 μm. Also, the particle size distribution of DS loaded matrix pellets was characterized by small span values, as these calculated values were found to be 0.64-0.72 indicating a narrow particle size distribution (22). Moreover, for each polymer grade, increasing the polymer concentration resulted in a decrease in the calculated volume weighted mean as well as the span value of particle

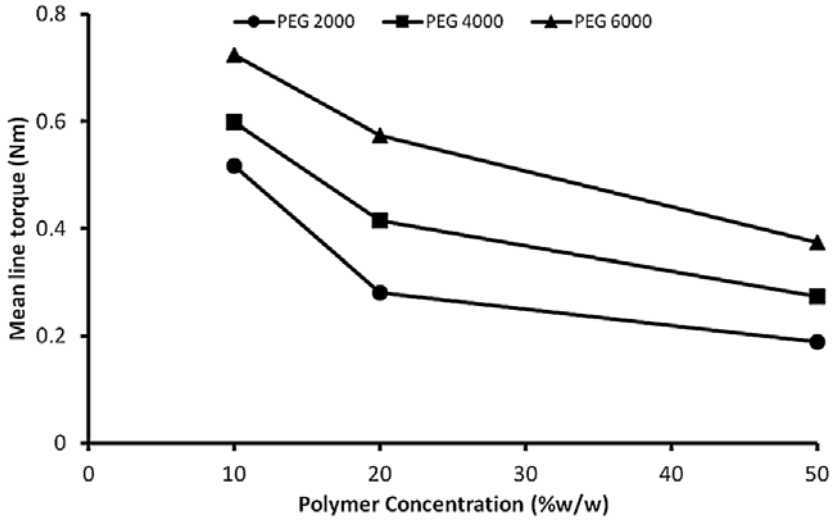

Figure 4. Effect of PEGs molecular weight and concentration of on mean line torque of Avicel PH101

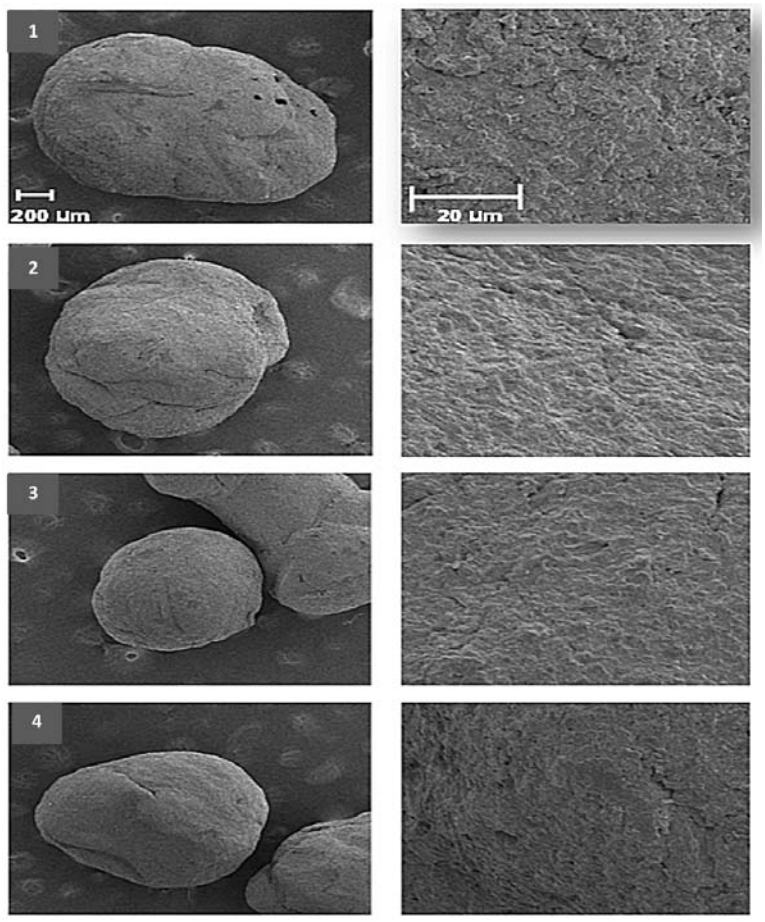

Figure 5. A column represents scanning electron micrographs of the pellets and B column represents scanning electron micrographs of the surface of pellets. Key: 1. Avicel only, 2. Avicel + 50% PEG 2000, 3. Avicel + 50% PEG 4000 and 4. Avicel + PEG 6000

size distribution. This is in accordance with the data obtained from wet massing studies; which showed a decrease in the wet mass by increasing polymer concentration, which in turn, reduced torque values. Kristensen and Schaefer (23) found a linear correlation between the torque value and pellet size for formulations containing 80% (w/w) of MCC.

Scanning electron micrographs of matrix pellets formulations containing 50% of each PEG grade mixed with avicel are compared with those prepared using avicel only and displayed in Figure 5. Most of the prepared pellets formulae were seen almost rounded and intact in shape, while pellets from avicel only (A) were not completely spherical. The higher torque value of this pellet wet mass formula (943) may be contributed to its irregular shape. Also, pellet formula prepared using 50% PEG 2000 (B) showed smooth surface compared to those prepared from 50% PEG 4000 (C) and PEG 6000 (D). Increasing PEG molecular weight caused increased roughness of the pellet surface, which might be due to increasing pellet wet mass mean torque as previously described. These results are in accordance with the data obtained by Mahrous et al. (13), who showed that the more hydrophilic polymer (PEG 4000), when mixed with MCC, produced a wet mass having the lowest mean torque value compared to that recorded with the same weight ratio of PVP and HPMC. This in turn, reflects on the easy extrusion of PEG wet mass resulting in pellets with smooth less rough surfaces. In addition, Law and Deasy (24) showed that the use of hydrophilic polymers with MCC favored more spherical and smooth pellets.

Table 2. Volume weighted mean particle size and the d(0.1), d(0.5), d(0.9) and span values of different pellet formulae loaded with 5% w/w diclofenac sodium (as determined by laser diffractometry).

Pellet formulations	Mean (µm)	d (0.1) µm	d (0.5) µm	d (0.9) µm	Span value
Avicel only	1065.74	741.52	1110.21	1541.87	0.72
Avicel + 10% PEG 2000	1030.24	734.21	1084.21	1498.21	0.71
Avicel +20% PEG 2000	1000.21	711.51	1051.21	1421.84	0.68
Avicel +50% PEG 2000	993.21	684.21	1000.10	1327.21	0.64
Avicel +10% PEG 4000	1075.11	721.45	1121.11	1524.32	0.72
Avicel +20% PEG 4000	1063.21	711.25	1101.01	1499.17	0.72
Avicel +50% PEG 4000	1033.45	700.14	1042.11	1418.71	0.69
Avicel +10% PEG 6000	1120.04	765.21	1132.10	1548.15	0.69
Avicel +20% PEG 6000	1086.21	751.78	1123.34	1513.01	0.68
Avicel +50% PEG 6000	1084.51	738.41	1108.91	1465.87	0.66

Table 3. Kinetic modeling of DS release from different sustained release matrix pellet formulations.

Formula	Zero order model	First order model	Higuchi diffusion model	Peppas model	n*
	r	r	r	r	
Avicel only	0.869	0.922	0.974	0.981	0.398
Avicel + 10% PEG 2000	0.957	0.970	0.999	0.999	0.50
Avicel +20% PEG 2000	0.869	0.981	0.974	0.981	0.398
Avicel +50% PEG 2000	0.819	-	0.948	0.960	0.366
Avicel +10% PEG 4000	0.869	0.938	0.975	0.981	0.398
Avicel +20% PEG 4000	0.879	0.965	0.978	0.984	0.41
Avicel +50% PEG 4000	0.872	0.967	0.976	0.982	0.399
Avicel +10% PEG 6000	0.869	0.930	0.974	0.981	0.398
Avicel +20% PEG 6000	0.870	0.952	0.973	0.978	0.41
Avicel +50% PEG 6000	0.855	0.948	0.966	0.972	0.394

r = correlation coefficient, and n is the release exponent. * obtained from Korsmeyer-Peppas equation.

***In vitro* release studies**

It was shown by Law and Deasy (24) that mixing various hydrophilic polymers with MCC had been reported previously to aid extrusion-spheronization and, at the same time, to enhance the dissolution of indomethacin. Therefore, the aim of studying DS *in vitro* release from matrix pellets is to investigate the effect of different PEGs on the drug release patterns. Incorporation of the drug in pellet formulations composed of MCC only resulted in slowing its release rate. Only 59% of the loaded DS was released from avicel matrix pellets after 8 h (Figs. 6-8). The effect of PEG 2000 concentration on the *in vitro* release profile of DS from matrix pellets is illustrated in Figure 6. The drug release rate was found to be enhanced by increasing PEG 2000 level in the pellets. For example, complete drug release was observed after 4 h in case of pellet formulation containing 50% PEG 2000, while only 66% and 79% of the loaded drug were released from the formulae containing 10 and 20% of such polymer at the same time, respectively. In case of pellet

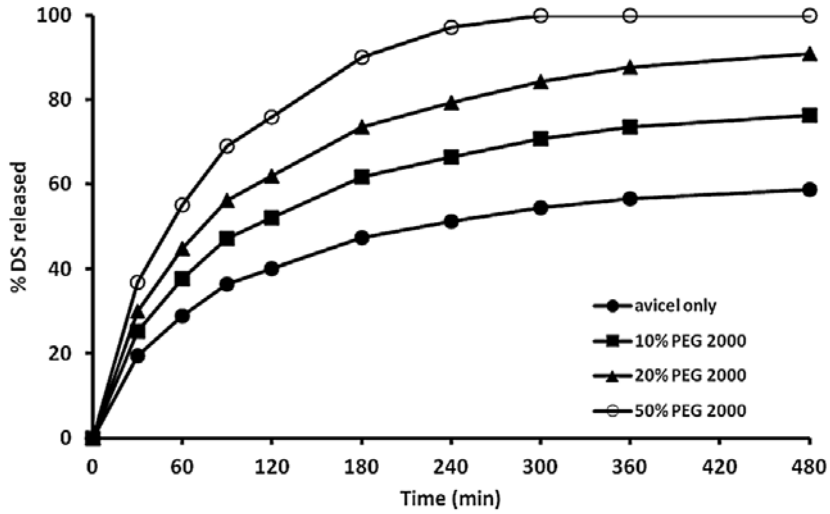

Figure 6. Effect of PEG 2000 concentration on the in vitro release profiles of DS from matrix pellets

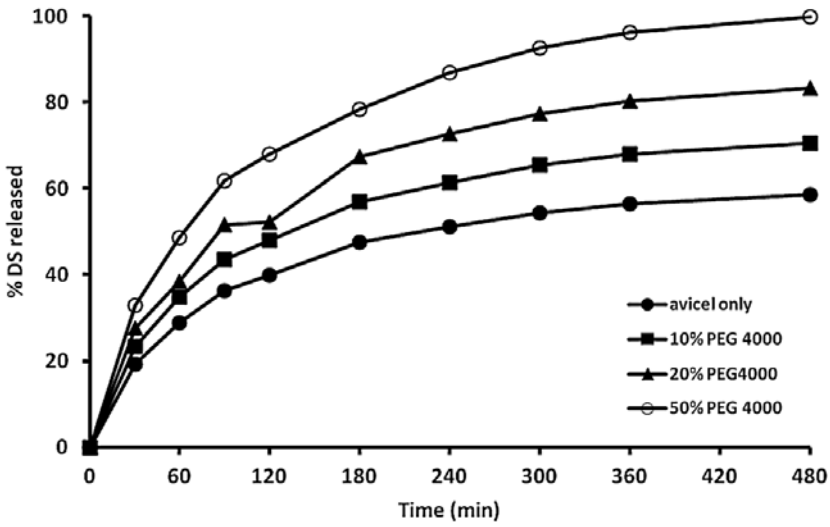

Figure 7. Effect of PEG 4000 concentration on the in vitro release profiles of DS from matrix pellets

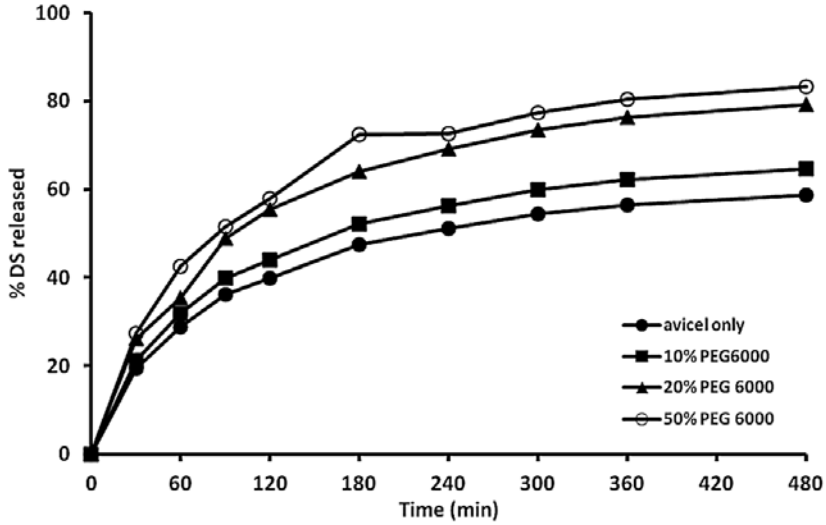

Figure 8. Effect of PEG 6000 concentration on the *in vitro* release profiles of DS from matrix pellets

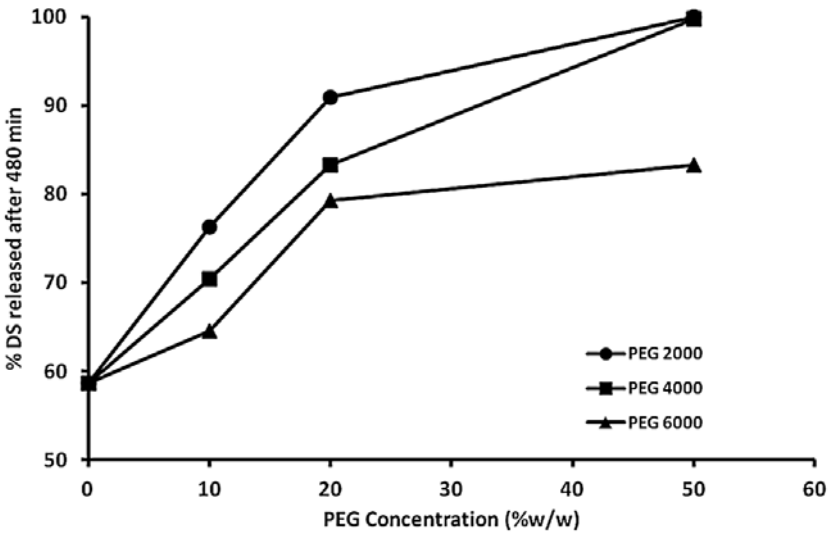

Figure 9. Effect of PEGs molecular weight and concentration on the release rate of DS from matrix pellets after 480 min

formulations containing PEG 4000 (Fig. 7) similar finding were recorded that by increasing PEG 4000 level in the pellet formulation, a pronounced rapid release rate was observed. However, the enhancement of DS release was in case of using different PEG 4000 concentrations lower that that exhibited by PEG 2000. Only 71, 83 and 99% of the loaded DS were released after 8 h from pellet formulations manufactured by using PEG 4000 concentrations of 10, 20 and 50% of the pellets' weight. Moreover, the addition of PEG 6000 in different levels caused an increase in the drug release rate by increasing PEG 6000 level (Fig. 8) but the enhancement is rather smaller than that seen in case of PEG 2000 and PEG 4000. For example, pellet formulations containing PEG 6000 concentrations of 10, 20 and 50% of the pellets' weight released 66, 79 and 83% of the loaded DS after 8 h.

Figure 9 correlates the effect of PEG molecular weight and level on the percentage of DS released

after 480 min. It is clearly evident that increasing PEG level in the pellet formula caused a decrease in the peak torque of wet mass, which in turn, enhanced DS release rate from pellet formulations. In addition, the effect of PEG 2000 and PEG 4000 on the drug release rate from pellet formulas is more noticeable than that exhibited by blending PEG 6000, especially at higher concentrations (20 and 50%).

In another study, Ibrahim (12) revealed that increasing lactose weight ratio was accompanied by enhancing the mefenamic acid release rate from matrix pellets by reducing pellet wet mass peak torque. He showed that lactose enhances the drug release rate by forming pores; it also promotes water penetration into the formulation core. In addition, increasing lactose concentration caused a pronounced lowering of the mean torque of pellet wet mass before extrusion/spheronization procedures. Also, Ibrahim et al. (25) found an inverse relationship between indomethacin release from its loaded pellets and the peak torque values of the polymer mixed with co-solvents.

Kinetic modeling of the *in vitro* release of MA from the matrix pellets

The *in vitro* release data of DS from different sustained release matrix pellets were fitted using zero order, first order and Higuchi diffusion models as well as Korsmeyer-Peppas equation to determine the best model that describes drug release from pellet formulations. Preference of the best release mechanism is based on the correlation coefficient value. The data revealed a good fit to Higuchi diffusion model. Successive evidence of the relative validity of diffusion model was obtained by analyzing the data using the equation of Korsmeyer et al., and the release exponent (n) was calculated from Korsmeyer equation: (26)

$$Mt/M_{\infty} = K. t^{n}$$

where Mt/M_{∞} is the fraction released by the drug at time t, K is a constant incorporating structural and geometric characteristic and n is the release exponent characteristic for the drug transport mechanism. For spherical samples, when n = 0.43 Fickian diffusion is observed and the release rate is dependent on t, while $0.43 < n < 1.0$ indicates anomalous (non Fickian) transport and when n = 1, the release is zero order.

The release kinetic parameters listed Table 3 indicated that the calculated n values were found mostly less than 0.45, indicating the so called non-Fickian or anomalous drug release or the coupled diffusion/polymer relaxation. Other investigators showed that when liquid diffusion rate and polymer relaxation rate (erosion) are of equal magnitude, anomalous or non-Fickian diffusion is observed (27, 28).

CONCLUSION

Diclofenac sodium was successfully prepared as sustained release matrix pellets using extrusion/spheronization technique. The results showed that the release of DS from matrix pellets can be tuned by controlling PEG molecular weight, which affects the rheological properties of pellets' wet masses. Mix torque rheometry was found to be a good tool for characterizing pellets' wet mass prior to extrusion/spheronization procedures. In addition, formulation of drug-loaded matrix pellets might be an alternative approach for pellet coating to avoid coating procedures drawbacks.

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OLANZAPINE-PEG 6000 BINARY SYSTEMS: *IN VITRO* DISSOLUTION BEHAVIOR, PHYSICOCHEMICAL CHARACTERIZATION AND MATHEMATICAL MODELING

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Abstract: The objective of the present work is to study the dissolution behavior of olanzapine from its solid dispersions with PEG 6000. Solid dispersions were prepared by melt dispersion method and characterized by phase solubility studies, drug content and *in vitro* dissolution studies. The best releasing dispersions were characterized by X-ray diffraction, differential scanning calorimetry, FT-IR spectroscopy, Near Infrared, Raman analysis and wettability studies. The phase solubility studies and its thermodynamic parameters indicated the spontaneity and solubilization effect of the carrier. The release study results showed greater improvement of drug release from solid dispersions than pure drug and a linear increase in drug release was observed with an increase in carrier content. XRD, DSC, FT-IR, NIR and Raman analysis revealed the crystallinity reduction of olanzapine and its compatibility with the carrier. Wettability studies proved the increased wettability in samples due to water absorbing nature of the carrier. The possible mechanisms for increased release rate are attributed to solubilization effect of the carrier, formation of solid solution, prevention of agglomeration or aggregation of drug particles, change in surface hydrophobicity, increased wettability and dispersability of drug in dissolution medium. The suggested reasons for such release behavior were found to be well supported by results of the evaluation techniques.

Keywords: olanzapine, PEG 6000, solid dispersions, X-ray diffraction, wettability, solid solution

Drug dissolution from solid oral dosage forms depends on the release of drug from the dosage form and subsequent release of drug in physiological fluids. It has been estimated that nearly 35-40% of drugs suffer from poor aqueous solubility and this affects the absorption of drug from gastrointestinal tract that leads to poor oral bioavailability, high intra and inter subject variability, increase in dose, reduction in therapeutic efficiency and finally, failure in formulation development (1). Development of solid dosage forms for water insoluble drugs had been a major challenge for pharmaceutical scientists for decades. Various formulation strategies like micronization, micellar solubilization, complexation, dendrimers for drug solubilization, formation of solid solutions/dispersions with hydrophilic carriers, self emulsifying drug delivery systems, spray drying, nano approaches, pro-drug approaches and salt synthesis have been developed to increase the dissolution rate of these types of drugs (2).

An attractive possibility could be represented by employing simple solid dispersion technique utilizing various hydrophilic carriers. Solid dispersions (SDs) are defined as the dispersion of one or more active ingredients in an inert hydrophilic carrier or matrix in a solid state, prepared by the fusion, solvent or solvent-fusion method. This technique provides a means of reducing particle size to a nearly molecular level, offers a variety of processing and excipients options that allow for flexibility when formulating oral delivery systems of poor water soluble drugs with cost effectiveness and significant dose reduction. It has been widely demonstrated that hydrophilic carrier dissolves rapidly exposing the drug particles to dissolution medium as fine particles for quick dissolution and absorption (3).

The mechanisms for increased dissolution rate may include reduction of crystallite size, a solubilization effect of the carrier, absence of aggregation of drug crystallites, improved wettability and dis-

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persability of a drug from the dispersion, dissolution of the drug in the hydrophilic carrier or conversion of the drug to an amorphous state (3).

Schizophrenia is a severe, non-curable illness of brain with serious consequences if not properly treated and kept under control. Olanzapine (2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno-[2,3-*b*],[1,5]benzodiazepine; OLZ) is a relatively new benzodiazepine atypical antipsychotic which belongs to the class of thienobenzodiazepines and has proven efficacy against the positive and negative symptoms of schizophrenia, bipolar disorder and other psychosis. It is poorly water soluble drug and belongs to BCS class II drug (low solubility and high permeability) and highly bound to plasma protein (about 93%). Following oral administration, C_{max} is reached within 5-6 h of dosing. OLZ undergoes extensive pre-systemic metabolism in liver resulting in relatively very low oral bioavailability (4-6).

MATERIALS AND METHODS

Materials

OLZ was received as a gift sample from Unichem Laboratories, (Mumbai, India). Mannitol was purchased from SD Fine Chemicals Ltd., (Mumbai, India). Sodium hydroxide, potassium dihydrogen orthophosphate, microcrystalline cellulose (DC grade) and magnesium stearate were purchased from SD Fine Chemicals Ltd., (Mumbai, India). All other solvents and reagents used were of AnalaR grade.

Melt dispersion method

SDs containing OLZ were prepared using varying concentrations of PEG 6000 and keeping CLZ concentration constant. The drug : carrier ratios used were 1 : 1, 1 : 2, 1 : 4, 1 : 6, 1 : 8 and 1 : 10. The required amount of carrier was melted in a china dish and weighed amount of the drug was added to molten carrier with constant stirring. The mixture was solidified to get waxy mass and dried in desiccators for 12 h. The hardened mixture was powdered in a mortar, sieved through a 100 mesh screen and the dispersion was wrapped and stored in dessicator (7-10).

Phase solubility studies

Phase solubility studies were carried out by adding excess amount of drug to 25 mL of aqueous solutions containing increasing amounts of carrier (1 : 1 - 1 : 10) in screw capped bottles and shaken in orbital shaker (Remi Ltd., Mumbai) incubated at 25 and 37°C for 24 h. Samples with pure OLZ and water were used as control. After 24 h, the solutions were filtered using filter paper (0.45 µm, 13 mm, Whatman, USA). The filtrate was diluted and analyzed spectrophotometrically at 259 nm (1700 UV-Vis Shimadzu, Japan). The solubility of OLZ in various carriers was calculated using the standard curve [OD = 0.1149 × concentration - 0.0031]. The data were subjected to phase solubility analysis to calculate various thermodynamic parameters like ΔH, ΔS and ΔG (11-13).

Phase solubility analysis

Stability constant (11-13)

The value of apparent stability constant, Ka between drug–carrier combinations were computed from the phase solubility profiles as described below from Eq. (1)

$$Ka = \frac{Slope}{Intercept\ (1 - Slope)} \quad (1)$$

Gibbs free energy, ΔG was calculated from Eq. (2)

$$\Delta G = -RT \ln Ka \quad (2)$$

where R - gas constant, 8.313 J/mol K, T - temperature, Ka - stability constant.

Enthalpy

The enthalpy change in systems was calculated from Eq. (3)

$$\Delta H = \frac{-RT \ln Ka}{dT\ (K)} \quad (3)$$

where, R - gas constant (8.313 J/mol K), Ka - stability constant, dT - difference in temperature (Kelvin).

Entropy

The entropy of the system was calculated from Eq. (4)

$$\Delta S = \frac{\Delta H - \Delta G}{T} \quad (4)$$

where ΔH - enthalpy, ΔG - entropy.

Drug content

Assay of weighed amount of SDs were carried out to determine the drug content. The weighed samples were dissolved in 10 mL of analytical media and stirred by vortex mixer. The solutions were filtered, using Whatman filter paper (0.45 µm, 13 mm, Whatman, USA). Next, the filtrate was diluted suitably and the content was estimated spectrophotometrically (UV-1700, Shimadzu, Japan) at 259 nm using standard curve.

In vitro dissolution studies

Dissolution of olanzapine (20 mg), and all SDs in PEG 6000 (equivalent to 20 mg of OLZ), was car-

ried out using USP dissolution test apparatus (Type II) at a temperature of 37 ± 0.5 °C, at 100 rpm using 900 mL 0.1 M HCL as dissolution medium. Five mL sample was withdrawn at 5.0, 10, 20, 30, 40, 50 and 60 min. The withdrawn sample was replenished with 5.0 mL of fresh media. The withdrawn samples were analyzed for OLZ content by measuring the absorbance at 259 nm using UV-visible spectrophotometer (UV-1700, Shimadzu, Japan). Three such determinations were carried out for each formulation. The content of olanzapine was calculated from the standard curve [OD = 0.1149 × concentration + 0.001 (R^2 = 0.9999; p > 0.001). The *in vitro* dissolution parameters namely, cumulative percent drug release, dissolution parameters like amount released (Q), percent dissolution efficiency (% DE), dissolution rate constant (DRC), relative dissolution rate (RDR), dissolution half life ($t_{50\%}$) and time taken to release 85% of drug ($t_{85\%}$) were calculated by subjecting the release data into various equations given below (12-16).

Dissolution half life ($t_{50\%}$)

Time taken to release 50% of drug was calculated by using Eq. (5)

$$t_{50\%} = \frac{0.693}{K} \quad (5)$$

Relative dissolution rate (RDR)

It is the ratio of the drug released from the samples with respect to pure drug at specific time intervals.

Dissolution efficiency (% DE)

It can be defined as the area under the dissolution curve up to a certain time. It is measured using the trapezoidal method and is expressed as a percentage of the area of the rectangle divided by the area of 100% dissolution in the same time and calculated from the Eq. (6)

$$\%DE = \left(\frac{\int_0^t y\,dt}{y_{100} \times t}\right) 100 \quad (6)$$

Dissolution rate constant (DRC)

A plot of log % drug unreleased *versus* time was drawn and the slope was calculated using MS Excel 2007 computer programme. Dissolution rate constant was calculated from Eq. 7

$$DRC = Slope \times 2.303 \quad (7)$$

Release kinetics

In order to describe the kinetics of drug release from the preparations, various mathematical equations have been proposed (16-18). The zero-order equation (Eq. 8), the first-order equation (Eq. 9), and the Higuchi model (Eq. 10), Hixson-Crowell model (Eq. 11) and Korsmeyer-Peppas (Eq. (12) were used in the present study

Zero order:

$$Q_t = Q_0 + K_o t \quad (8)$$

where Q_t = amount of drug released at time t, Q_0 = amount of drug in solution at time t = 0, (usually Q_0 = 0) and K_o = zero order release constant.

First order:

$$\log Q_t = \log Q_0 \times K_1 \frac{t}{2.303} \quad (9)$$

where Q_t = amount of drug released in time t, Q_0 = amount of drug in solution at time t = 0, (usually Q_0 = 0) and K_1 = first order release constant.

Higuchi model:

$$M_t = K\sqrt{t} \quad (10)$$

where M_t = amount of drug dissolved at particular time "t", K – Higuchi release constant.

Hixson Crowell model:

$$(W_0)^{1/3} - (W_t)^{1/3} = k_{1/3}t \quad (11)$$

where W_0 = weight of the drug taken at time t = 0 and W_t = weight of the drug taken at time "t"

Korsmeyer-Peppas empirical model:

$$\frac{Q_t}{Q_\infty} = k_{KP} \times t^n \quad (12)$$

Where, Q_t/Q_∞ = fractional release of drug at time t, k_{KP} = a constant comprising the structural characteristics of the formulation and "n" (the release component) = a parameter indicative of the mechanism of drug release. For the particular case of delivery system, n = 0.5 corresponds to Fickian release (case I), 0.5 < n < 1.0 to an anomalous (non Fickian) transport, n = 1 to a zero order release kinetics (case II) and n > 1 to a super case II transport (17-19).

Solid state characterization

X-ray diffraction studies (X-RD)

X-ray diffractometer (Philips, Finland) consisting of 40 kV, 30 mA generator with a Cu-Kα radiation tube was used. Diffraction patterns of pure drug (PD), physical mixtures (PM) and selected SDs were scanned over 2θ range from 2° to 50° at the rate of 2° per min at 0.02° at 2θ step size.

Differential scanning calorimetry (DSC) studies

Thermal analysis was carried out using differential scanning calorimeter (Q 10 DSC TA, Instruments, Waters Inc., Newcastle, USA) with liquid nitrogen cooling accessory. The analysis was performed under purge of nitrogen gas (50 mL/min). High purity indium was used to calibrate the heat flow and heat capacity of the instruments. Sample (5-10 mg), placed in flat bottomed aluminum pan,

was firmly crimped with lid to provide an adequate seal. Sample was heated from ambient temperature to 400°C at preprogrammed heating rate of 10°C/min.

Fourier Transform Infrared (FT-IR) spectroscopic studies

FT-IR spectra of pure OLZ, carriers, physical mixtures of drug and carrier (1 : 1) and optimized SDs were carried out using FT-IR spectrophotometer with KBr disc (Jasco - FT-IR 1700 spectrophotometer, Japan). All the samples viz. OLZ, mannitol and physical mixtures (PMs) and SDs were analyzed in similar manner. Physical mixtures were prepared by blending individual component in glass-pestle mortar.

Near infrared (NIR) analysis

NIR spectra of pure drug and selected samples were recorded in FT-IR spectrometer (Jasco FT-IR, Japan) in diffuse reflectance mode (DRS). The samples were scanned in the wavelength range of 800 – 2000 nm and absorbance was measured in transmittance mode.

Raman spectroscopic analysis

The Raman spectra of samples and pure drug were recorded in Confocal Raman spectrophotometer [WITEC Alpha 300, Confocal Raman Nd: YAG laser (532 nm), USA].

Drug polymer miscibility studies

Miscibility of the drug with the polymer can be assessed based upon the shift in melting endotherm or T_g of the drug or can be predicted theoretically using the following Gordon-Taylor equations (Eqs. 13 and 14) based on the T_g, densities, and weight fractions of the components.

$$T_{gmix}(SDS) = \frac{W_1 T_{g1} + \kappa W_2 T_{g2}}{W_1 + \kappa W_2} \quad (13)$$

$$\kappa = \frac{T_{g1}\rho_1}{T_{g2}\rho_2} \quad (14)$$

where, T_{g1} is the glass transition temperature of drug, W_1 and W_2 are the weight fractions of the components, and κ is the parameter calculated from the true densities (ρ_1 of drug and ρ_2 of polymer) and T_{g2} of the amorphous dispersions. The true densities of OLZ and the carrier were determined in a duplicate using a pycnometer (20-22).

Flory-Huggins (F-H) modeling

The Flory-Huggins model can be applied to calculate the interaction parameter (x) by using Eq. 15

$$\frac{1}{T_m\text{mix}} - \frac{1}{T_m\text{pure}} = \frac{-R}{\Delta H_f}\{\ln\phi_{Drug} + (1 - \frac{1}{m})\phi_{Polymer} + \phi^2_{Polymer}\} \quad (15)$$

where, T_m mix is the melting temperature of the drug in the presence of the polymer, T_m pure is the melting temperature of the drug in the absence of the polymer, ΔH_f is the heat of fusion of the pure drug, m is the ratio of the volume of the polymer to OLZ, and ϕ drug and ϕ polymer are the volume fractions of the drug and the polymer, respectively (20-22).

Wetting studies

Formulation of tablets

The tablets of pure OLZ and selected SDs were formulated by using 20 mg of pure drug and SDs equivalent to 20 mg of OLZ and the formulation scheme is shown in Table 1. Sufficient quantity of microcrystalline cellulose (diluent) and magnesium stearate (lubricant) were added and mixed well in a mortar. The mixture was directly compressed in a 10 station rotary tablet punching machine (Rimek, Ltd., Mumbai, India) at a compression pressure of 5 kg/cm^2. Each tablet weighed around 250 mg.

Wetting time studies

Five circular tissue papers were placed in a Petri dish of 10 cm diameter. Ten mL of water containing 0.5% methylene blue, a water-soluble dye, was added to the Petri dish. The dye solution was used to identify complete wetting of the tablet surface. A tablet was carefully placed on the surface of the tissue paper in the Petri

Table 1 Formulation scheme of tablets for wetting studies.

Composition	Olanzapine (mg)	OPEG10 (mg)
OLZ	20	–
Selected SD (OPEG10)	–	= 20 mg of olanzapine (217 mg of SD)
Microcrystalline cellulose	223	26
Magnesium stearate	7	7
Total	250	250

dish at ambient temperature. The time required for water to reach the upper surface of the tablets and to completely wet them was noted as the wetting time. These measurements were carried out in replicates of three. Wetting time was recorded with digital watch (23, 24).

Water absorption ratio (23, 24)

The weight of the tablet prior to placement in the Petri dish was noted (W_b), utilizing a Mettler Toledo digital balance. The wetted tablet was removed and reweighed (W_a). Water absorption ratio R, was then determined using Eq. (16)

$$R = \frac{W_a - W_b}{W_b} \times 100 \qquad (16)$$

***In vitro* dispersion studies**

A tablet was added to 10 mL of phosphate buffer pH 7.4 at 37°C. The time required for complete dispersion was noted. Three such determinations were carried out (25).

Statistical analysis

The relevance of difference in the *in vitro* dissolution profile and pharmacokinetic parameters were evaluated statistically. The data were tested by two way analysis of variance.

RESULTS

Physicochemical characterization

Phase solubility studies

Phase solubility studies were conducted to determine the effect of temperature, solubilization effect of carrier and the spontaneity of solubilizing process when the drugs is physically mixed with PEG 6000. The thermodynamic parameters of OLZ and its PMs are shown in Table. 2. The solubility of OLZ was found to show a linear increase with an increase in amount of carrier and temperature. The thermodynamic parameters like ΔG and ΔH were found to be negative and entropy ΔS of physical mixtures was positive in nature.

Drug content

The assayed drug content in all SDs was in the range of 98-104%.

In vitro dissolution studies

The % cumulative release of pure OLZ was found to be 70% in 1 h while SDs showed a significant improvement in release rate in the same period. The *in vitro* release profiles of the dispersions were compared in Figure 1. The *in vitro* dissolution

Table 2. Thermodynamic parameters of olanzapine physical mixtures with PEG 6000.

	Carrier	Temp °C	Slope	Intercept	Ka (M^{-1})	Δ G (kJ/mol)	Δ H (kJ/mol)	Δ S (J/mol K)
1.	PEG 6000	25	231.37	-7.338	0.137	-2.841	-2.841	2.831
2.		37	191.10	-4.029	0.245	-3.307	-3.308	3.296

Table 3. Dissolution parameters of olanzapine-PEG 6000 solid dispersions.

Code	Composition OLZ : PEG	Q05[a] (mg)	Q30[b] (mg)	%DE[c]	RDR[d] 05	RDR[d] 30	DRC[e]	$t_{50\%}$[f] (min)	$t_{85\%}$[g] (min)
OLZ	1 : 0	9.34 (0.12)	12.15 (0.56)	59.61	-	-	0.020	12.5	> 60
OPEG1	1 : 1	12.03 (0.24)	13.52 (1.24)	65.75	1.29	1.11	0.022	4.5	> 60
OPEG2	1 : 2	12.08 (0.24)	14.09 (0.40)	68.28	1.29	1.16	0.021	4.5	> 60
OPEG4	1 : 4	13.51 (0.20)	14.27 (0.39)	70.10	1.45	1.17	0.021	3.5	> 60
OPEG6	1 : 6	14.48 (0.31)	15.84 (0.28)	76.78	1.55	1.30	0.017	3.5	60
OPEG8	1 : 8	14.61 (0.32)	15.89 (0.57)	77.06	1.56	1.31	0.017	3.5	60
OPEG10	1 : 10	14.95 (0.31)	16.23 (0.54)	79.75	1.60	1.34	0.010	3.0	43.5

Values in parenthesis indicate standard deviation. [a]Q_{05} – Amount released at 05 min (mg); [b]Q_{30} – Amount released at 30 min (mg); [c]% DE – % Dissolution efficiency; [d]RDR - Relative dissolution rate at specific time intervals; [e]DRC - Dissolution rate constant; [f]$t_{50\%}$ - Dissolution half- life; [g]$t_{85\%}$ - Time taken to release 85% of drug from dispersions.

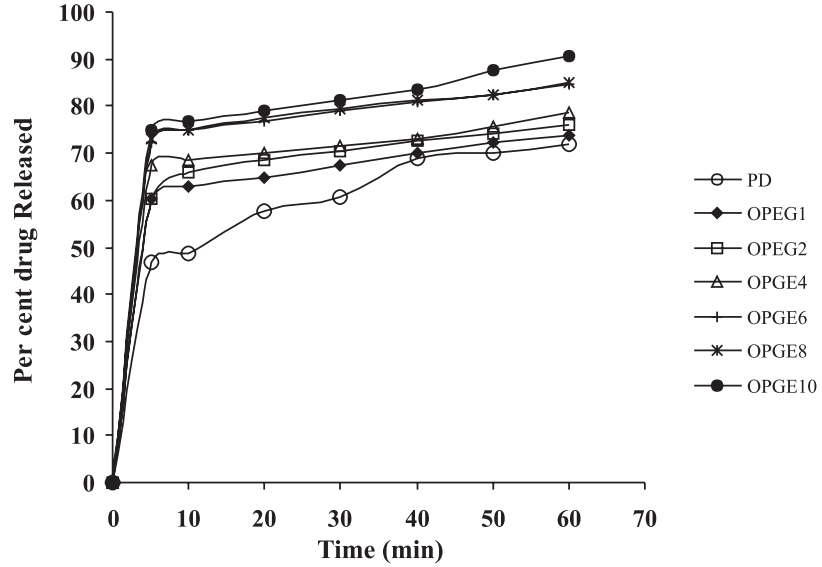

Figure 1. Dissolution profiles of olanzapine-PEG 6000 SDs compared with pure drug. All data points represent the mean of 3 values, n = 3

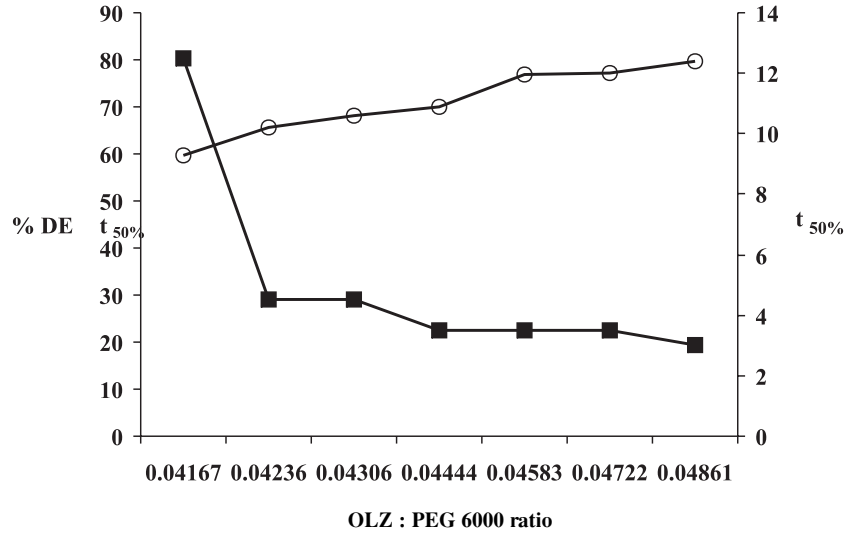

Figure 2. Relationship plot of % dissolution efficiency (% DE) and dissolution half life profiles of olanzapine-PEG 6000 solid dispersions. ■ - t_{50} %, ○ - % DE

parameters of the SDs are presented in Table 3. The relationship plot of % DE and $t_{50\%}$ was shown in Figure 2.

Release kinetic analysis

The release kinetics of the *in vitro* dissolution data (Table 4) and the regression parameters were analyzed to ascertain the type of drug release from SDs.

Solid state characterization

The best releasing dispersions (OPEG10) among the samples were subjected to solid state characterization

X-ray diffraction analysis

X- ray diffraction spectra of pure OLZ, PEG 6000, physical mixture (1 : 1) compared with the best releasing dispersion (Batch OPEG10) are illustrated in Figure 3.

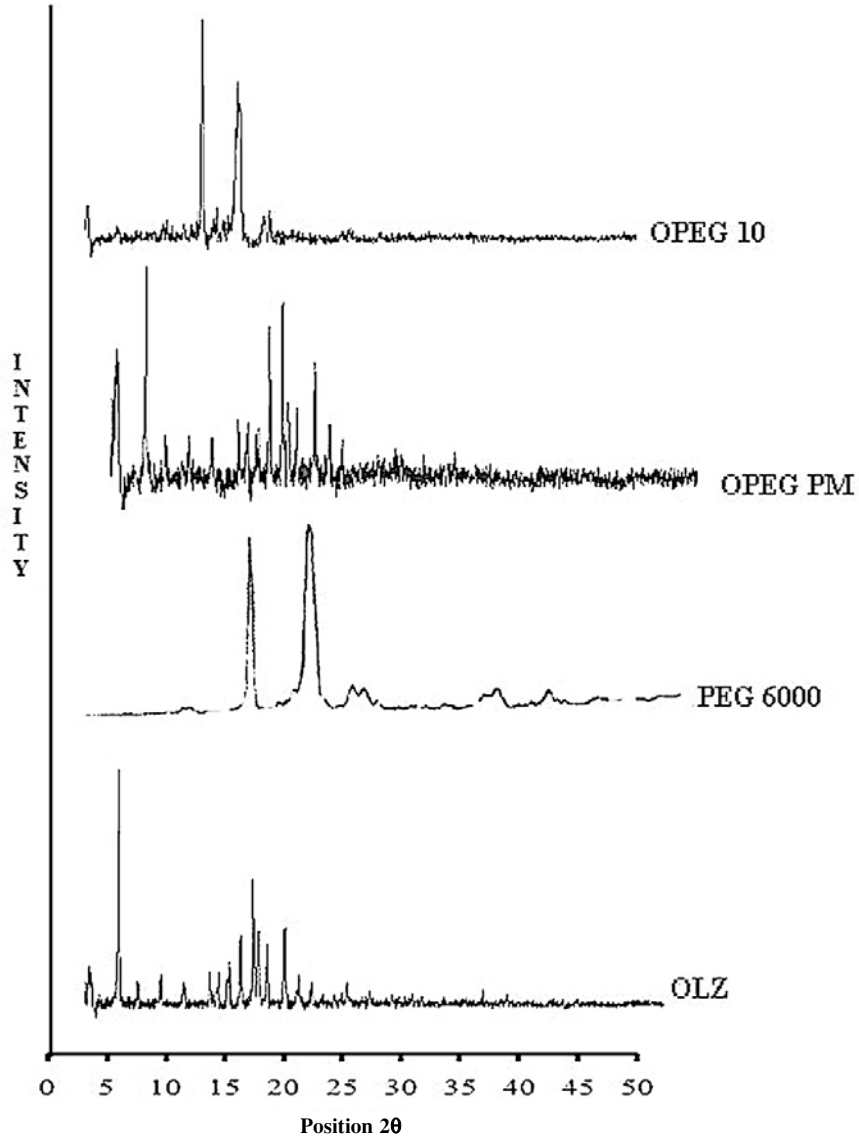

Figure 3. X-RD spectra of pure olanzapine (OLZ), PEG 6000, physical mixtures (PM) at 1 : 1 ratio and solid dispersions (SDs) OPEG at 1 : 1 ratio

Table 4. Release kinetic parameters of olanzapine – PEG 6000 solid dispersions.

Code	Zero order		First order			Higuchi		Hixson Crowell		K-P	
	r^2	K_0	r^2	Slope	K_1	r^2	Slope	r^2	Slope	r^2	"n"
OLZ	0.796	0.998	0.110	0.009	0.020	0.857	8.240	0.751	0.012	0.993	0.257
OPEG1	0.653	0.725	0.154	0.009	0.021	0.677	7.585	0.521	0.010	0.951	0.255
OPEG2	0.650	0.749	0.144	0.009	0.021	0.679	7.880	0.521	0.011	0.953	0.265
OPEG4	0.612	0.720	0.156	0.009	0.021	0.622	7.709	0.460	0.010	0.930	0.265
OPEG6	0.614	0.790	0.114	0.007	0.016	0.630	8.476	0.489	0.012	0.935	0.290
OPEG8	0.610	0.788	0.115	0.007	0.016	0.626	8.476	0.483	0.012	0.933	0.291
OPEG10	0.643	0.866	0.044	0.004	0.009	0.661	9.091	0.586	0.015	0.944	0.307

K-P – Korsmeyer-Peppas model, " n" – release exponent

Table 5. Gordon-Taylor analysis data.

Code	D : C ratio	W_1	T_{g1} (°C)	$W_1 \times T_g$	W_2	T_{g2} (°C)	$W_2 \times T_{g2}$	ρ_1	ρ_2	$T_{g1} \times \rho_1$	$T_{g2} \times \rho_2$	κ	$W_1 \times T_{g1} + W_2 \times T_{g2}$	$W_1 + \kappa_{w2}$	T_g Mix (°C)
OPEG10	1 : 10	1	196.4	196.4	10	14.96	149.6	1.3	1.08	255.32	16.15	15.8	2560.47	159.0.	16.10

T_{g1} - glass transition temperature of drug; W_1 and W_2 -weight fractions of the components; κ - parameter calculated from the true densities (ρ_1 of drug and ρ_2 of polymer); T_{g2} – Experimental glass transition temperature of the dispersions ; T_g Mix – Predicted glass transition temperature of the dispersion

Table 6. Flory-Huggins modeling data.

Code	D : C ratio	T_{pure}	T_{mix}	$1/T_{mix}$	$1/T_{pure}$	ΔH_f	$-R/\Delta H_f$	OLZ	m	ln ϕ drug	(1-1/m) ϕ polymer	ϕ^2 polymer	χ
OLZ	1 : 0	196.4	-	-	0.005	105	-0.079					0	
OPEG10	1 : 10	196.4	14.96	0.066	0.005	9.07	-0.916	1	10	2.718	0	100	-0.0074

R - 8.314; $T_{m\ mix}$ – melting temperature of the drug in the presence of the polymer; $T_{m\ pure}$ – melting temperature of the drug in the absence of the polymer; ΔH_f – Heat of fusion of the pure drug; m – ratio of the volume of the polymer to OLZ; ϕ drug and ϕ polymer are the volume fractions of the drug and the polymer; χ - interaction parameter.

DSC studies

The DSC scans of pure OLZ, PEG 6000 and optimized SDs (OPEG10) are compared in Figure 4.

FT-IR studies

The FT-IR spectra of OLZ, PMs (1 : 1) and SDs in PEG 6000 are presented in Figure 5.

Near infra red analysis

The near infrared spectra of OLZ and optimized SDs (OPEG10) are compared in Figure 6.

Raman analysis

The Raman spectra of pure OLZ and selected SDs (OPEG10) are compared in Figure 7.

Drug polymer miscibility studies

Gordon-Taylor analysis

The results of Gordon Taylor analysis are shown in Table 5.

Flory-Huggins (FH) modelling

The results of the FH modeling analysis are shown in Table 6.

Wettability studies

The wettability data of pure OLZ and optimized SDs (OPEG10) are shown in Table 7.

DISCUSSION

Phase solubility studies

These results were found to be in accordance with the well established formation of weak soluble complexes (11-13). It was also stated that the drug molecules might have transferred from pure water into the aqueous solution of carriers, which was indicated clearly from the negative values. These findings prove the spontaneous nature of the solubilization process. The enhancement of drug solubility in hydrophilic carrier could also be equally well explained by co-solvency effect of the carrier. It was also suggested that the hydrophilic carriers may interact with the drug molecules by electrostatic bonds and other types of forces like Van der Waals forces and this would have lead to the formation of weakly soluble complexes. The slopes of straight linear relationship assumed as indicative of the relative solubilizing efficiency of the carrier (11-13).

Drug content

The drug content values in the SDs indicate the uniform distribution of the drug in formulation and the suitability of the method used for formulation.

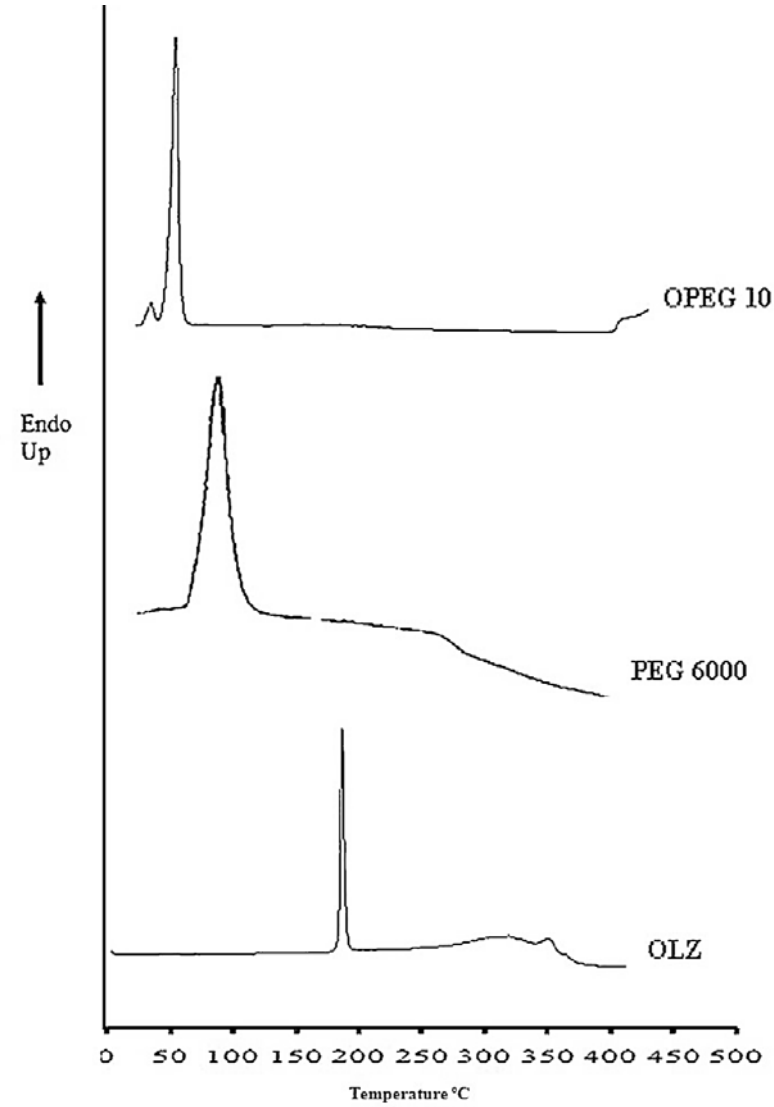

Figure 4. DSC thermograms of pure olanzapine (OLZ), PEG 6000 and solid dispersions (SDs) at 1 : 10 ratio

***In vitro* release analysis**

The percentage of drug release from SDs was found to increase gradually as the amount of carrier in SDs was increased from 1 : 1 to 1 : 10 (Fig. 1). The *in vitro* release data of sample SDs showed significant difference ($p < 0.001$) in release rate in comparison with pure OLZ.

It was found that dissolution parameters like per cent cumulative release, amount of drug released, % DE and RDR values were found to exhibit a linear increase with an increase in the amount of PEG 6000 in SDs. The other parameters like DRC, $t_{50\%}$ and $t_{85\%}$ values tend to decrease with an increase in carrier fraction.

The % DE values were found to increase from 59.61% (for pure OLZ) to 79.75% (for SDs with 1 : 10 ratio). The dissolution half life was found to decrease from 12.5 (pure OLZ) to 3.0 (for OPEG6-10) and $t_{85\%}$ were found to be reduced from 60 min (for pure OLZ) to 43.5 min (for OPEG6-10). Based on these findings, it can be inferred that batch OPEG10 was identified as the best releasing batch than other SDs. The order of OLZ drug release from the SDs could be ranked as: OPEG10 > OPEG8 > OPEG6 > OPEG4 > OPEG2 > OPEG1 > OLZ.

The relevance of difference in $t_{50\%}$ and % DE were evaluated statistically. When examined by two way analysis of variance, the $t_{50\%}$ and % DE data

showed significant difference between the pure OLZ and test products ($p < 0.001$). However, within the tests products a significant difference was not observed indicating that the data of all SDs differ significantly. Hence, it can be inferred that samples are not the same but are different in their formulations.

The possible reasons for enhanced release rate of OLZ from such SDs could be attributed to the modification of hydrophobic surface properties of OLZ due to the formation of film of polyethylene glycol around the drug particles and increased wettability of the powder with the dissolution medium (26).

The factors like decreased particle size, decreased crystallinity and prevention of aggregation and agglomeration of the drug by the carrier,

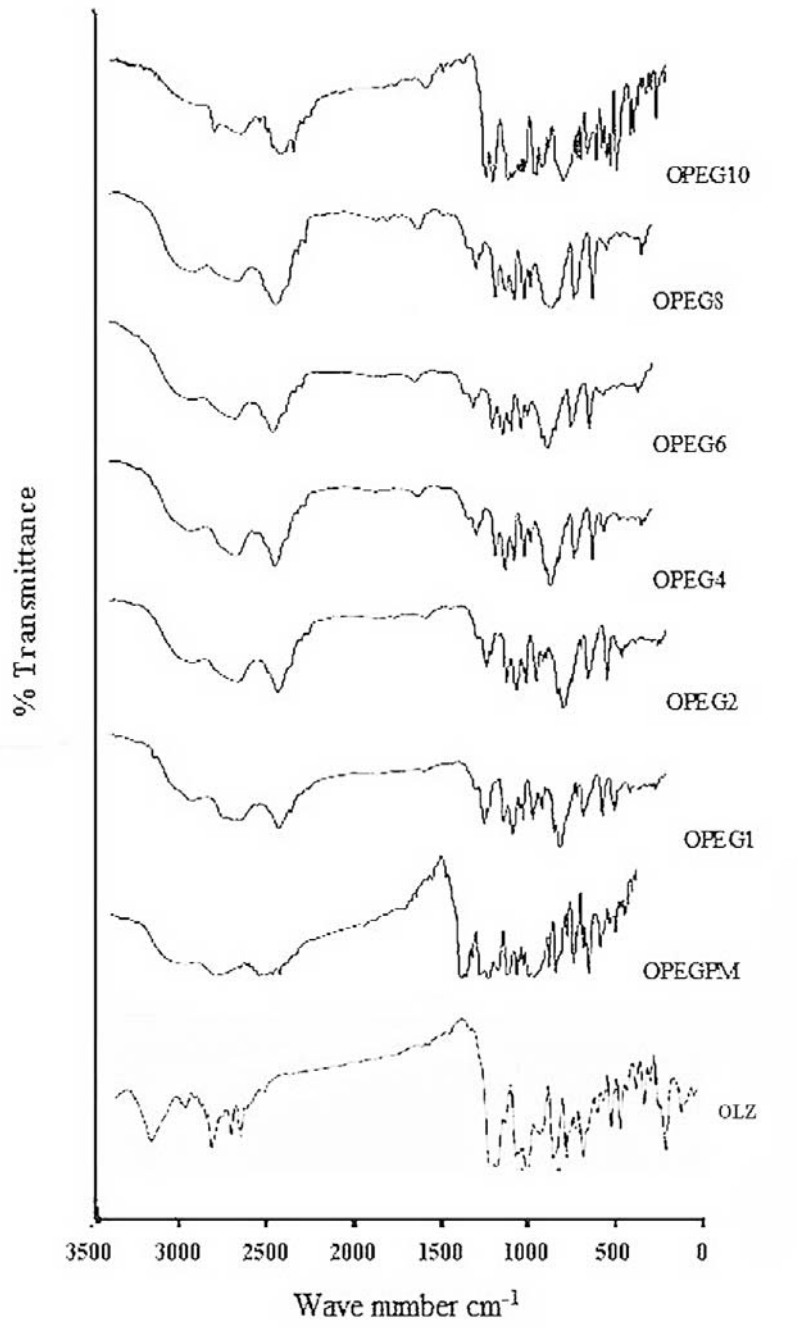

Figure 5. FT-IR spectra of pure olanzapine (OLZ), physical mixtures (PM) at 1 : 1 ratio, solid dispersions (SDs) OPEG1, OPEG2, OPEG4, OPEG6, OPEG8 and OPEG10

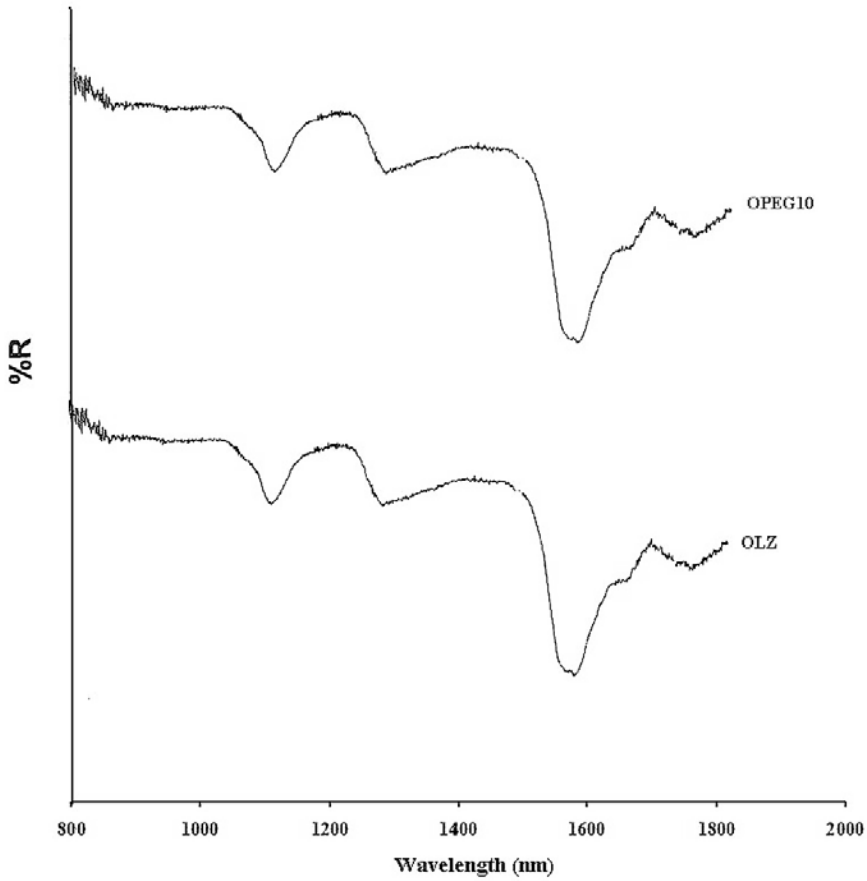

Figure 6. Near infrared spectra of pure olanzapine (OLZ) and solid dispersions (SDs) OPEG10

formation of solid solution are also indicated as the additional factors behind the enhanced dissolution rate from the SDs (8-10, 26-28).

Further, the method of formulation would also decide about the nature of the drug in carrier structure. It was noticed that most of the dispersions formulated by melt solvent or melt dispersion method using PEG as carriers had resulted in formation of a solid solution i.e., drug being molecularly dispersed in the carrier structure. The above said postulations was well supported from the observations made from the results of the *in vitro* wettability and dispersion studies which clearly showed that a polymer rich layer was formed around the tablet and diffusion was clearly observed and tablets surface was found to decrease gradually during the dissolution process (26-28).

Release kinetic analysis

From the release kinetic data of the dispersions it was noticed that the coefficient of correlation "r" value of Korsmeyer Peppas model was found to predominate over the "r" value in other models the release data were found to fit aptly in to Korsmeyer-Peppas kinetic model. Further, the release exponent "n" values were found to be well within 0-0.5, suggesting a Fickian type of drug release from dispersion. The possible mechanisms suggested for high release of OLZ from dispersions was also found to correlate with the findings of release kinetic analysis (14-19).

Solid state characterization

X-ray diffraction analysis

X- ray diffraction spectra of pure OLZ, PEG 6000, physical mixture (1 : 1) and batch OPEG10 are illustrated in Figure 3. The presence of numerous distinctive sharp intense peaks at 2θ of 8.79, 18.48 with peak height of 758.62 and 142.4 in diffractogram of OLZ indicates its high crystalline character (29-31). The carrier (PEG 6000) spectra exhibited a distinct diffraction pattern with two prominent

peaks indicating its nature. The principal peaks of OLZ were found to appear in diffractogram of physical mixture (1 : 1) ratio, suggesting the absence of interaction between drug and carrier.

The peaks present in sample diffractogram are also owed to the carrier PEG 6000. The absence of crystalline peaks of pure OLZ in sample diffractogram indicates that the drug was molecularly dispersed in the carrier structure (26-28).

DSC studies

A sharp single endothermic peak appeared for pure OLZ with the following parameters; Onset at 194.36°C, peak at 196.40°C, area of 262.56 mJ and DH value of 105.023. These values clearly indicate its high crystalline nature (29-31).

A single broad endothermic peak at 65°C in PEG 6000 thermogram denotes its low melting point and its amorphous nature. It was also observed that the prominent peak of OLZ was completely absent in sample (OPEG10) thermogram. Further, the peak parameters (onset at 39.35°C, peak at 45°C, peak area of 28.14 mJ and DH value of 9.078) of samples thermogram in comparison with pure drug may all be related to the nature of carrier. The absence of characteristic peaks of OLZ in sample thermogram clearly reveals that the drug was no more present in its undissolved form and would have resulted in formation of solid solution in dispersions (11, 12, 15). This postulation was also well supported by the method used for preparation of the dispersions with PEG 6000 as carrier, since the drug was added to the molten carrier and solidified. It was also widely reported that many drugs had formed solid solution or dispersed in molecular form, when dispersed in carrier like PEG 6000 (29-31).

FT-IR studies

From the IR spectra of pure drug, carrier and the SDs it was noticed that pure olanzapine showed characteristic absorptions at 3239 cm^{-1} (NH and OH stretching), 2929 cm^{-1} (C-H stretching), 1587 cm^{-1} (C=C stretching), 1421cm^{-1} (C=N stretching) and 1287 cm^{-1} (C-N stretching). The characteristic peaks of pure OLZ were found to be present in the spectra of PM as well as in SDs. This finding reveals the lack of interaction between drug and the carrier in samples. It was also noticed that the significant peaks of pure OLZ at specific wave number (3239 cm^{-1}) was found to be reduced gradually in sharpness and increased in broadness as the amount of PEG 6000 was increased in samples. These findings clearly reveal the possibility of formation of solid solution of drug in the carrier (32).

Near infrared analysis

The characteristic peaks of pure OLZ appeared at 1141 nm and 1581 nm (29, 30). The specific peaks of OLZ were found to be broader in nature and a slight shift in the peak position in spectra of optimized SDs was observed. These findings indicate the reduction of crystallinity of drug present in SDs.

Raman analysis

The sharp peaks of OLZ appeared at 2435, 1594, 1517, 1460, 1224, 1050, 965, 784 and 480 cm^{-1} positions which indicated its high crystallinity (29, 30). The characteristic peaks of pure OLZ were found to be in much reduced form with broadness and slight shift toward their lower wave numbers in sample spectra. These findings clearly suggest that some degree of structural changes had taken place in the drug molecule when dispersed in hydrophilic carriers.

Drug-polymer miscibility studies

Gordon-Taylor analysis

Incomplete miscibility or reduced solubility can result in the formation of concentrated drug spheres that may lead to recrystallization after production and during stability. The T_g of pure OLZ was found to be 196°C and the carrier PEG 6000 showed a T_g of 52°C. A single T_g was observed for the optimized solid dispersions. According to the Gordon-Taylor equation, if the drug and polymer are miscible, the binary mixture will exhibit a single T_g as shown in Table 4. The predicted T_g was found

Table 7. Wettability data of pure olanzapine and selected solid dispersions.

Batch	Wettting time (min)	Water absorption ratio	*In vitro* dispersion time (min)
OLZ	> 60 (2.26)	11.49 (1.14)	> 60 (1.12)
OPEG10	22 (1.24)	15.20 (0.84)	22 (0.96)

Values in parentheses indicate standard deviation n = 3.

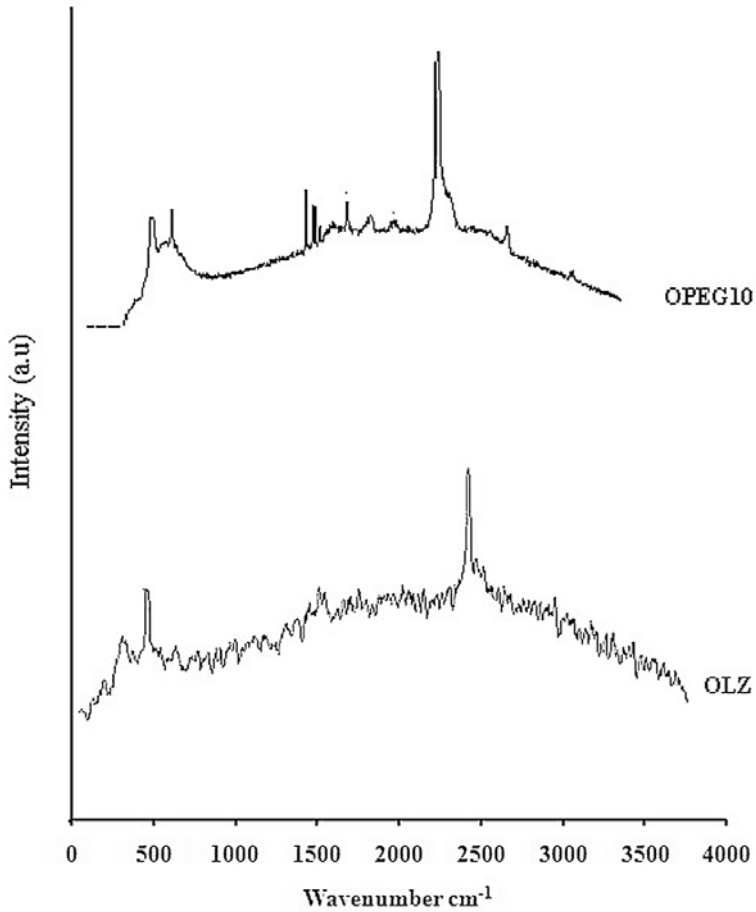

Figure 7. Raman spectra of olanzapine and SDs (OLZ and OPEG10)

to be closely related to the experimental values of the optimized dispersions. These findings were found to correlate well with the earlier reports (20-22).

Flory-Huggins (FH) modeling

FH modeling suggests that if the interaction parameter $\chi \geq 0.5/M$, there are slightest possibility for unfavorable interactions between the drug, polymer and excipient mixture, which may cause phase separation. It was noticed that the calculated value of FH interaction factor (χ) (Table 5) for the optimized dispersions was found to be $\geq 0.5/M$, which signifies higher favorable extent of drug-polymer interactions at micro level. This behavior may be attributed to the reduction of entropy during the formulation of solid dispersion and it also indicated the thermodynamic stability of developed SDs. Adhesive interaction between drug and polymer was favored by the reduction in the T_g of SDs, which implicates the miscibility of drug and polymer (20-22).

Wettability studies

The wetting time and *in vitro* dispersion of pure OLZ was found to be more than 60 min and water absorption ratio of OLZ was found to about 11.49. It was observed that tablets prepared with olanzapine did not show any sign of structural changes after 60 min and it was also found to retain its compactness during the *in vitro* dispersion studies. These results clearly prove the high hydrophobicity, poor wettability and low water absorption potential of OLZ (25-28). The wetting time and *in vitro* dispersion time of sample was found to be 22 min, much less than the pure OLZ (more than 60 min). The water absorption ratio of sample was found to be higher (15.20) than pure OLZ (11.49) indicating the increased water absorption by PEG 6000. It was also observed that tablets prepared with samples of

OPEG10 showed a slow and steady rate of absorption of water and the size of the tablet gradually decreased in size as the time proceeded and it showed the formation of polymer rich layer around the tablet surface resulting in fine powder with smooth appearance. These observations confirm the increased wettability in samples and it also provides a clear insight into the role of hydrophilic carriers in formulations (23-25).

Mechanisms for enhanced release

The possible reasons that might have attributed for increased release rate from SDs are proposed and summarized as particle size reduction, solubilization effect of carrier, change in crystal quality, or formation of solid solution, prevention of aggregation or agglomeration of drug particles in dissolution medium, change in surface hydrophobicity of drug particles, increased wettability due to increased water absorption by the carrier. These postulations were well supported by findings of various physicochemical characterization techniques used for evaluation of SDs. Further, the suggested mechanism for enhanced release was found to be in accordance with the earlier published reports of using such hydrophilic carriers (3, 7-13, 26-29, 33, 34).

CONCLUSION

The findings from this work provide a clear insight into the drug release enhancement process from such systems. The results of the work clearly suggest that SDs formulated with PEG 6000 could be developed in fast release dosage forms with improved oral absorption and therapeutic efficiency. The SDs could be explored further to establish pharmacokinetic and pharmacodynamic profiles to utilize their potential.

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The authors declare that there is no conflict of interest among them.

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PHARMACOLOGY

IMPACT OF ISOPRENALINE AND CAFFEINE ON DEVELOPMENT OF LEFT VENTRICULAR HYPERTROPHY AND RENAL HEMODYNAMIC IN WISTAR KYOTO RATS

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Abstract: Left ventricular hypertrophy (LVH) is a compensatory mechanism in response to an increased work load on the heart. This study investigated the impact of chronic isoprenaline and caffeine (I/C model) administration on cardiac geometry, systemic hemodynamic and physiological data in rats as LVH develops. LVH was induced by administering isoprenaline (5 mg/kg s.c. every 72 h) and caffeine (62 mg/L) in drinking water for 14 days to Wistar Kyoto (WKY) rats. Mean arterial pressure (MAP), systolic blood pressure (SBP), heart weight, LV weight, LV chamber diameter and thickness of myocardium were observed as LVH indicators. MAP was significantly higher (142 ± 13 *vs.* 119 ± 2 mmHg, respectively) while heart rate (HR) in LVH was lower (314 ± 9 *vs.* 264 ± 18 BPM) compared to control WKY. Heart weight, LV weight and kidney weight were 31%, 38% and 7%, respectively, greater in the LVH group as compared to the control WKY (all $p < 0.05$).The myocardium thickness was 101% greater while LV chamber diameter was 44% smaller in the LVH group as compared to the control WKY ($p < 0.05$). The superoxide dismutase (SOD), glutathione reductase (GSH) and total antioxidant capacity (T-AOC) levels were significantly reduced while malonodialdehyde (MDA) level increased in LVH as compared to control WKY (all $p < 0.05$). In conclusion, isoprenaline and caffeine (I/C) induces LVH and cardiac hypertrophy with increases in blood pressure, fluid excretion and reduced renal hemodynamics. Prooxidant mechanism of the body and arterial stiffness are dominant in this disease model. This model of LVH is easily generated and associated with low mortality.

Keywords: isoprenaline, caffeine, left ventricular hypertrophy, hemodynamics, renal function

Left ventricular hypertrophy (LVH) is defined as an increase in mass and size of the left ventricle which frequently occurs as a result of an elevated resistance within the circulatory system. This increase in mass of the myocardium results from a chronically raised workload on the heart (1) and is taken as an early sign of cardiomyopathy, which ultimately may lead to symptomatic heart failure. LVH is an independent predictor of cardiovascular morbidity and mortality (1, 2). There are two types of LVH: (i) pressure overload hypertrophy (POH) or, (ii) volume overload hypertrophy (VOH). LVH has been studied using different animal models based on catecholamine administration, for example, isoprenaline, caffeine, torbafylline, as well as a combination of isoprenaline with caffeine (I/C) (3) and isoprenaline with torbafylline (4) for varying periods up to 7 days. Isoprenaline given as an intraperitoneal injection (i.p.) of 5 mg/kg/day for 7 days has been used as a model for heart failure (5). A further variation has been when isoprenaline was administered for the development of myocardial necrosis in which 2 injections of isoprenaline (s.c.) were used some 72 h apart (6).

The left ventricular hypertrophy and altered cardiac output will impact on systemic hemodynamic and the function of other organs, including the kidney. The functional capacity of renal adrenergic receptors in the kidney has been studied using the I/C model where treatment lasted for 7 days, which probably induced a degree of cardiac hypertrophy, and combined with diabetes in Sprague-Dawley

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(SD) rats (7). This study demonstrated that α_{1A} and α_{1D} subtypes mediate adrenergically induced vasoconstriction responses in this combined state of heart failure and diabetes. In another I/C model study, caffeine was administered as 62 mg/L while isoprenaline was injected (s.c.) at the dose of 5 mg/kg for 14 days in anesthetized Wistar Kyoto rats (3). The investigators reported that although cardiac function was not impaired in LVH, there was decreased high pressure baroreceptor control of sympathetic outflow. These investigators went on to show that in the same I/C model as well as one in which heart hypertrophy was induced by giving thyroxin (1 mg/kg, s.c.) for 1 week (8) there was a defective baroreceptor regulation of renal sympathetic nerve activity. These reports found no significant difference in baseline value of blood pressure, heart rate or RSNA in vehicle control, isoprenaline/caffeine-treated or thyroxin-treated groups of anesthetized animals. However, following an acute saline volume expansion in these models, the renal sympatho-inhibition was prevented. This model was presumed to be one of a transition from LVH to heart failure (HF).

Metabolic changes are likely to occur during the induction of LVH and an increased plasma level of angiotensin II has been observed in catecholamine induced LVH (9). In the I/C model, both isoprenaline and caffeine act as cardiac stimulants. Caffeine is an adenosine receptor antagonist and increases heart rate along with activation of noradrenergic neurons (10). This increased adrenergic activity will affect the renal hemodynamic and excretory functional capacity of kidney.

Left ventricular remodelling is a multifactorial process with the involvement of inflammatory cytokines and production of reactive oxygen species (11), oxidative stress and inflammatory reactions in injured myocardium (12). It is there important to know the balance between prooxidant and antioxidant. Plasma levels of oxidative stress parameters (SOD), malonodialdehyde and nitric oxide (NO) levels should be altered in LVH. To the best of our knowledge, these parameters of oxidative stress have not been studied yet in this isoprenaline and caffeine model of LVH and cardiac hypertrophy.

Based on previous investigations, the current study used a modification of the original isoprenaline and caffeine regime to induce LVH by extending isoprenaline therapy to achieve consistent cardiac dysfunction. This study also aimed to investigate the impact of LVH on circulatory status, cardiac geometry, and systemic hemodynamic and oxidative stress parameters. This was extended to examine how blood perfusion through kidney was altered in LVH and to determine whether endothelial status had been altered as reflected in arterial stiffness.

MATERIALS AND METHODS

Animals

Fourteen male Wistar Kyoto rats (WKY) weighing 200 ± 20 g, were brought from the Animal Facility of Universiti Sains Malaysia (USM) and were acclimatized in the transit room for 5 days before starting any treatment. Animals were given free access to water and food. Animals were randomly divided into control and LVH groups. The control group and LVH groups were given the same food for 14 days while in the LVH group on day 1, the animals began treatment with isoprenaline (5 mg/k g s.c., 72 h apart) and caffeine (62 mg/L) in the drinking water until day 14 as stated by (3) when they were taken for acute study. According to previous study (3), isoprenaline s.c. 4 injections were administered after 72 h apart but present study used 5 injections of isoprenaline 72 h apart on day 1, 4, 7, 10 and 13 to prolong the effect of drug before acute experiment. Caffeine dose was continued as stated (3). Experimental procedures were approved by the Animal Ethical Committee of USM approval no. 2012/(76) (364).

Measurements of electrocardiogram (ECG) data

Animals were fastened overnight and then anesthetized with injection of pentobarbital sodium (Nembutal; CEVA, Lebourne, France) at a dose of 60 mg/kg, i.p. A 3 lead surface ECG recordings of all the animals were done with gold plated needles electrodes (ADInstruments, Sydney, Australia) as previously reported (13, 14).

Acute experiment

The experimental procedure and protocols for the acute studies were based on those previously published studies (15). Overnight fasted rats were anesthetized using pentobarbital (Nembutal; CEVA, Lebourne, France) at a dose of 60 mg/kg, i.p. A tracheal tube PP 240 (propylene tubing 240; Portex, UK) was inserted to facilitate ventilation. The left jugular vein was cannulated with PP-50 to allow maintenance doses of anesthetic to be given during the course of surgery. The right carotid artery was cannulated with PP-50 and connected to a pressure transducer (P23 ID Gould; Statham Instruments, Nottingham, UK), linked to a computerized data acquisition system (Power Lab; AD

Instrumentation, Sydney, Australia) to monitor blood pressure, heart rate, cyclic duration and mean arterial pressure (MAP). The cyclic duration is taken as the time interval between one cardiac cycle and second cardiac cycle. The left kidney was exposed *via* an abdominal midline incision and a laser Doppler probe (OxyFlow, ADInstruments, Australia Model No. ML191) was placed on the cortical surface of the kidney to measure cortical blood perfusion, expressed as blood perfusion units. The left iliac artery was cannulated with PP-50 tubing to allow recording of iliac blood pressure. The iliac cannula was connected to a pressure transducer which was attached to a Powerlab (ADInstruments) and *via* a side arm was attached to an infusion pump programmed to deliver saline (0.9% NaCl) at 6 mL/h. After stabilization for about 60 min, pulse wave velocity was measured by exposing the aorta and measuring the distance from the point of insertion of the carotid artery cannula to the insertion point of the iliac artery cannula (16, 17). The left ureter was cannulated with PP-10 tubing for urine collection. At the end of acute experiment, animals were euthanized by means of an overdose of pentobarbital. The weight of the heart, left ventricle and kidney were taken after careful isolation and removal from experimental animals. After weighing, left ventricle tissues were put in 10% formalin for histopathology study. The thickness of the myocardium and LV chamber internal diameter was estimated as described previously (18). Thickness was measured with a Vernier caliper just below the level of the mitral valve. These values were used to generate heart index, kidney index and LV index as follows:

$$\text{Heart index} = \text{Heart weight} / \text{body weight} \times 100$$

$$\text{Left ventricle index} = \text{Left ventricle weight} / \text{body weight} \times 100$$

$$\text{Kidney index} = \text{Kidney weight} / \text{body weight} \times 100$$

The study protocol is shown in Figure 1.

Measurement of creatinine and electrolytes in plasma and urine samples

Both groups had fluid intake and urine output measured using metabolic cages before and after treatment (day 0 and day 14). Water intake was measured by subtracting the remaining water in the bottle after 24 h from the initial total volume. Blood (1 mL) was drawn from the lateral tail vein, centrifuged at 10,000 rpm for 10 min using a centrifuge (Gallenkamp, UK) and the supernatant layer was taken as plasma. Sodium and potassium in the plasma was estimated using a flame photometer (Jenway Ltd. Felsted, Essex, UK) while creatinine in plasma and urine was estimated using a colorimetric method (19) and the samples were read using a 96 well microplate reader. Creatinine was measured from

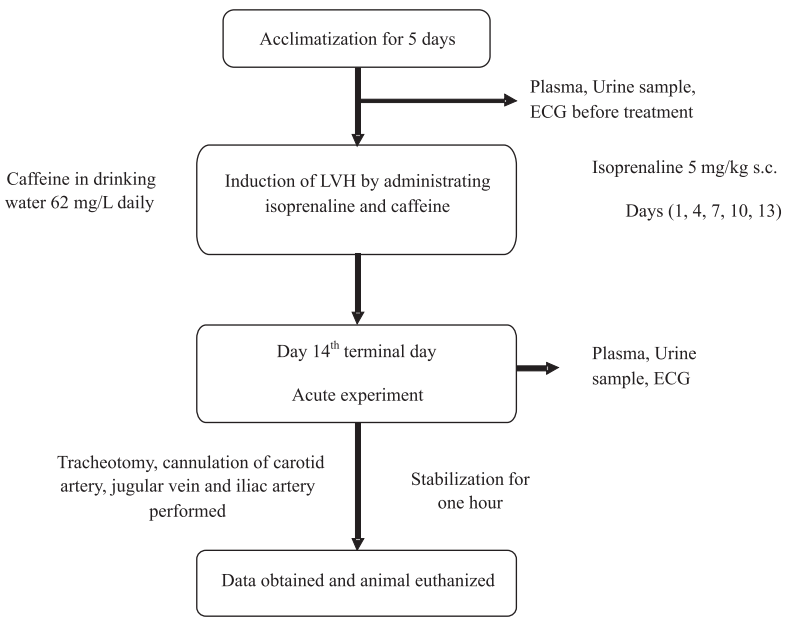

Figure 1. Study protocol conducted for validation and evaluation of left ventricular hypertrophy and hemodynamic parameters

plasma and urine to assess creatinine clearance (mL/min/kg b.w.) and fractional excretions of sodium and potassium (%).

Measurement of oxidative stress parameters

Oxidative stress parameters like superoxide dismutase (SOD), malonodialdehyde (MDA), glutathione reductase (GSH), nitric oxide (NO) and total antioxidant capacity were measured on day 14th from the plasma by using kits.

Statistical analysis and presentation of data

The data (the mean ± SEM) were analyzed using one way ANOVA along with the *post hoc* Bonferroni test and independent Student's *t* test using Graph Pad Prism (Graph Pad Software, Inc. CA, USA) with significance taken at $p < 0.05$.

RESULTS

Effect on systemic hemodynamic

The MAP, HR, PP and cyclic duration was observed principally to validate the model and use them as markers of LVH. MAP, PP and cyclic duration were significantly higher (all $p < 0.05$) in the LVH WKY group as compared to the control WKY (Table 1) while heart rate in the LVH WKY group was lower than in the control WKY group ($p < 0.05$) (Table 1).

Heart weight, left ventricle weight, kidney weight respective indices, myocardial thickness and LV chamber diameter

The physical appearance and weights of vital organs are primary indicators of LV and cardiac hypertrophy. Both heart and LV weights were significantly higher (both $p < 0.05$) in the LVH WKY group (Table 2) as compared to control WKY. The myocardium was thicker in the LVH WKY group compared to the control group while the internal diameter of left ventricles of the LVH WKY group was significantly less than that of the control group (Table 2, Fig. 2).

The electrocardiogram (ECG) measurement

The LVH was investigated by studying QRS complex, R-R intervals and R-amplitude in control WKY and LVH WKY rats by using ECG. The ECG morphology showed obvious P, Q, R and S waves in both groups. In this study QRS complex, R-R intervals and R-amplitudes were significantly higher in LVH WKY group as compared to control WKY (all $p < 0.05$) as shown in Table 1.

Table 1. Electrocardiogram of control WKY and LVH WKY done on day 14th during acute experiments. (n = 5-6). * denotes significant difference $p < 0.05$ compared to control group.

ECG	Control WKY	LVH WKY
R-R (ms)	175.7±3.9	211.7±6.*
QRS (ms)	18.6 ± 0.6	22.2 ± 0.9*
QT (ms)	72.5 ± 3.5	88.1 ± 1.2*
QTc (ms)	200.3 ± 3.1	210.0 ± 1.5*
R-Amp (mV)	0.60 ± 0.01	0.76 ± 0.03*

ms: milliseconds, mV: millivolts

Table 2. SBP, DBP, MAP, PP, and HR, cyclic duration, time to peak, of control WKY *vs*. LVH WKY groups (n = 5-6). * denotes significant difference $p < 0.05$ compared to control group.

Groups	Systolic blood pressure (mmHg)	Diastolic blood pressure (mmHg)	Mean arterial pressure (mmHg)	Pulse pressure (mmHg)	Heart rate (BPM)	Cyclic duration (S)
Control	132 ± 10	102 ± 4	119 ± 2	30 ± 2	314 ± 9	0.190 ± 0.0
LVH	159 ± 13*	125 ± 6	142 ± 13*	40 ± 7*	264 ± 18**	0.231 ± 0.03*

Abbreviations: LVH = Left Ventricular Hypertrophy; wt. = weight; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; MAP: Mean Arterial Pressure; PP: Pulse pressure; HR: Heart rate.

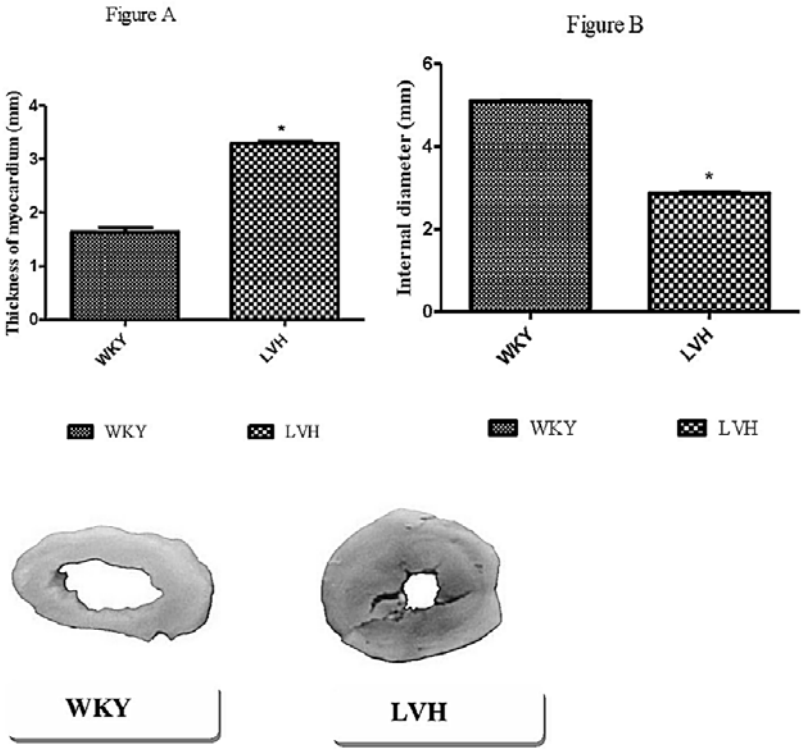

Figure 2. Thickness of myocardium, internal diameter of LV chamber of control WKY, LVH; * denotes significant difference $p < 0.05$ from the control group.

Table 3. Heart, left ventricle, kidney indices, myocardial thickness and diameter of LV at day 14th of LVH as compared to the control group. (n = 5-6). * represents mean value significantly different in comparison between control WKY and LVH WKY groups ($p < 0.05$).

Physical parameters	WKY	LVH
Body wt. (g)	298 ± 12	273 ± 8*
Heart wt. (g)	0.79 ± 0.02	1.04 ± 0.05*
LV wt. (g)	0.47 ± 0.02	0.64 ± 0.02*
Kidney wt. (g)	0.87 ± 0.02	0.93 ± 0.05
Heart index	0.26 ± 0.00	0.39 ± 0.01*
LV index	0.46 ± 0.03	0.68 ± 0.06*
Kidney index	0.30 ± 0.02	0.33 ± 0.02*
Myocardial thickness (mm)	1.64 ± 0.18	3.29 ± 0.10*
Diameter of LV (mm)	5.09 ± 0.04	2.85 ± 0.10*

Abbreviations: LVH = Left Ventricular Hypertrophy, wt. = weight

Effect on water intake and urine output in control and LVH group

Weight gain in LVH WKY group was significantly less compared to the control WKY group (Table 4a). Water intake and urine output in the two groups were not significantly different as shown in Table 4a.

Changes in physiological data in control and LVH groups

Plasma Na concentration of the control group was higher than that of LVH group ($p < 0.05$) while the concentration of Na in urine was significantly lower in the control group as compared to the LVH group (Table 4b). Plasma K^+ was lower in LVH

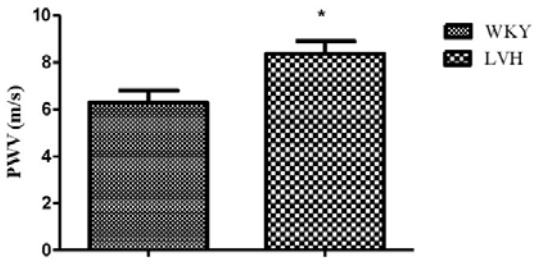

Figure 3. PWV of control *vs*. LVH. (n = 6 in each group) * represents $p < 0.05$ compared to control group

group as compared to control WKY group ($p < 0.05$) while urinary K^+ was higher in the LVH compared to the control WKY group (Table 4b). The U_{Na}/U_K ratio was significantly lower in the LVH ($p < 0.05$) compared to the control WKY group as shown in Table 4b. Absolute Na excretion and absolute potassium excretions in the control groups were significantly (both $p < 0.05$) lower than in the LVH group. Significantly elevated creatinine in plasma was observed in LVH WKY as compared to control WKY ($p < 0.05$). The fractional excretions of sodi-

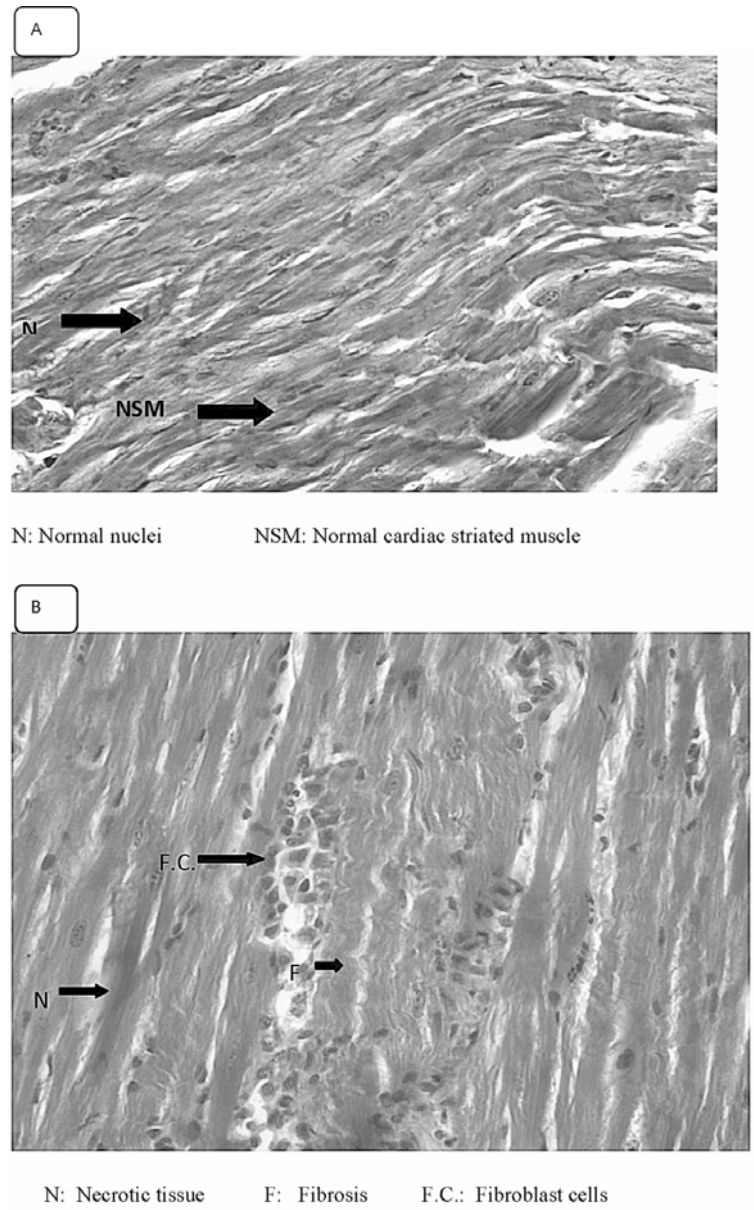

Figure 4. Histopathological slides of heart muscles of control WKY and LVH rats. Fig. A showing normal nuclei, no breakdown of muscle fibre and striated muscles while figure B showing the breakdown of myofibers , no cardiac muscle characteristic striation. Necrotic tissue seen and the presence of fibroblast and fibrosis observed in LVH WKY group

Table 4a. Body weight gain, daily water intake, daily urine output of control and LVH. * denotes significant difference $p < 0.05$ between control *vs*. LVH.

Groups	Wt. D-O	Wt. D-14	Wt. Gain	Water intake (mL/24 h)	Urine output (mL/24 h)
Control group	192	298 ± 5	106	45	10
LVH group	184	273 ± 3	89*	50	15*

Wt. represents weight, LVH is left ventricular hypertrophy and D represents day.

Table 4 (b). Electrolytes data of control WKY and LVH WKY groups. * denotes significant difference $p < 0.05$ in comparison of control WKY and LVH WKY.

Groups	Na in plasma (mmol/L)	K in plasma (mmol/L)	Na in urine (mmol/L)	K in urine (mmol/L)	Absolute Na excretion	Absolute K excretion
WKY	135 ± 3	6 ± 1.2	122 ± 7	60 ± 9.3	0.05 ± 0.01	0.14 ± 0.01
LVH	120 ± 3*	4 ± 1*	146 ± 9*	108 ± 5.1*	0.09 ± 0.01*	0.19 ± 0.02*

Na: Sodium; K: Potassium.

Table 4 (c). Renal functional parameters investigated on day 14th. * denotes significant difference $p < 0.05$ in comparison of control WKY *vs*. LVH WKY.

Renal function parameters	WKY	CONTROL
Creatinine in Plasma	1.05 ± 0.33	1.38 ± 0.03*
Cr.Cl (mL/min/kg b.w.)	1.14 ± 0.22	1.34 ± 0.10
FE Na (%)	2 ± 0.2 3	± 0.2*
FE K(%)	22 ± 4.5	79 ± 12.3*
Urine flow rate (µL/min/100 g b.w.)	2.38 ± 0.5	3.54 ± 0.3*
U_{Na}/U_K	2 ± 0.33	1.36 ± 0.10*

Cr.Cl: Creatinine clearance, b.w.: body weight, mL/min/kg: millilitre per minute per kilogram
FE Na: Fractional excretion of sodium; FE K: Fractional excretion of potassium; U_{Na}/U_K: Urinary sodium to urinary potassium ratio.

um, potassium and urine flow rate is significantly greater in LVH WKY group as compared to control WKY ($p < 0.05$) as shown in Figure 4c.

Oxidative stress parameters

In LVH disease model, the level of oxidative stress was measured as LVH is chronic inflammatory process. It was observed that enzymatic SOD, NO, GSH and T-AOC levels were significantly lower in LVH WKY group as compared to control WKY (all $p < 0.05$) while enzymatic oxidative parameter was higher in LVH WKY group as compared to control WKY ($p < 0.05$) as shown in Table 5.

Effect on pulse wave velocity (PWV)

Pulse wave velocity was significantly lower (6.3 ± 1.01 m/s) in control WKY as compared to LVH WKY (8.38 ± 1.04) indicating a higher arterial stiffness in LVH WKY group as shown in Figure 3.

Histopathology studies of left ventricles of Control WKY and LVH WKY

Histopathology observations are shown in Figure 4. The arrangement of the perimysial fibres in control (A, B) and LVH tissue (C, D) as can be seen with the straight arrows indicating the organized and uniform direction of these fibres while the circle in D slide shows distorted and deranged fibres which are indicative of collagen deposition.

Measurement of baseline value of renal cortical blood perfusion

The renal cortical blood perfusion was significantly lower in LVH WKY as compared to control

WKY indicating decreased blood perfusion in LVH WKY group ($p < 0.05$) local vasoconstriction in kidney as shown in Figure 5.

DISCUSSION

The present study was aimed at undertaking a detailed investigation into the cardiovascular and renal functional changes that develop during the chronic administration of isoprenaline and caffeine (I/C) to induce left ventricular hypertrophy (LVH). Moreover, a somewhat different regime of I/C administration was used like isoprenaline alone and in combination with methylxanthines (20) for 7 days. This I/C model was used for 2 weeks by giving 5 injections 72 h apart as we reported earlier (21) by modifying the original I/C model (4). Present studied modified this model by administering 5 inections of isoprenaline (5 mg/kg 72 h apart s.c.) and caffeine (62 mg/L) to make this model more valid for LVH and cardiac hypertrophy. Although previously reported data successfully described the LVH model but further modification definitely gave more confidence about reproducibility of data and model can be studied closer to heart failure. This model of LVH may be considered as a transition state between LVH and heart failure. Furthermore, a detailed examination was undertaken of the effect of I/C administration on cardiac geometry, especially the arrangement and breakdown of myofibers. A third area of consideration was the effect of I/C administration on renal and systemic hemodynamics including physiological parameters as reflected by electrolyte homeostasis, renal excretion, renal cortical blood perfusion and arterial elasticity.

Wistar Kyoto rats were selected for this study as SD rats have been reported to develop LVH spontaneously and are not considered to be an ideal strain to investigate LVH (22). A second reason for giving priority to the WKY strain is the high mortality during acute surgical procedures in SD rats which amounted to 80% in those rats which developed LVH (22). The original I/C model was modified by using 5 injections of isoprenaline (s.c.) over 2 weeks

Table 5. Oxidative stress parameters investigated on day 14th. * denotes significant difference $p < 0.05$ in comparison of control WKY *vs*. LVH WKY.

Parameters	WKY	CONTROL
SOD (µmol/mL)	5.51 ± 1	1.34 ± 0.1*
MDA (nmol/mL)	21 ± 6	34 ± 2*
NO (µmol/mL)	5 ± 1	21 ± 1
GSH (µmol/mL)	542 ± 43	134 ± 47*
T-AOC (µmol/L)	18 ± 3	10 ± 1*

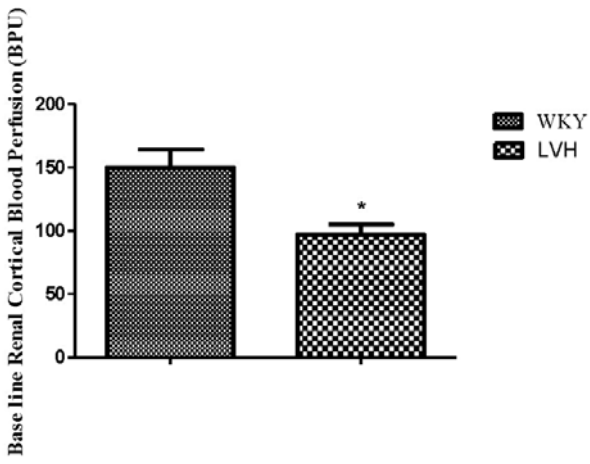

Figure 5. Comparison of renal cortical blood perfusion (RCP) in control and LVH (n = 6). *represents $p < 0.05$ compared with control group

that is 72 h apart (days 1, 4, 7, 10, 13), which was different from the 4 injections as previously reported (3). The current model was found to be safe not only during the treatment period but also over the long duration protocols of the acute surgery.

To the best of our knowledge, no previous study has exclusively investigated the impact of LVH on systemic hemodynamics in WKY rats using this I/C model. There was an elevation of systolic, diastolic and mean arterial pressure in the LVH compared to the control WKY group, as shown in Table 1, which supported the view that there was an activation of the sympathetic nervous system as a result of β_1 receptor stimulation. Our results are consistent with previous findings which suggested that LVH was due to activation of β ARs subtypes in this model (23). In fact, isoprenaline will activate both the β_1 receptors of heart and β_2 receptors in the peripheral vasculature (24). Caffeine is an adenosine receptor antagonist (25) and studies have shown that its administration will increase plasma levels of noradrenaline (26, 27). Both isoprenaline and caffeine will increase heart rate synergistically, which ultimately results in an elevation of systemic blood pressure (28). Many studies have shown elevated SBP, DBP, PP and MAP to be predictors of CV events in males (29). Pulse pressure was also elevated in the study, which also predicts LVH which is consistent with previous findings in man (30).

Surprisingly, heart rate in the LVH group was lower compared to the control WKY. This observation would be in line with previous findings generating cardiac hypertrophy models, in mice using isoprenaline (15 µg/g/day, s.c. for 7 days) (31), in SD rats using isoprenaline and methylxanthine (4) and in male Wistar Kyoto cardiac hypertrophied anesthetized rats by using I/C model (4 injections in 2 weeks) (3). A baroreceptor mediated reflex bradycardia could be the cause of the decreased heart rate in the LVH group. Noradrenaline in isolated tissue stimulates cardiac contractility while *in vivo*, when noradrenaline stimulates the heart, it increases heart rate and ultimately blood pressure. This rise in blood pressure induces a reflex rise in vagal activity by stimulating baroreceptors, resulting in reflex bradycardia, which could be sufficient to counteract the local action of noradrenaline on the heart (32). Down regulation of adrenoceptors under these conditions has been reported in some studies (33-35) but the exact mechanism responsible for the decreased heart rate remains unclear. The cyclic duration was increased in the LVH group consistent with a prognosis which may lead to heart failure.

The physical appearance and weight of key organs like the kidney and heart showed cardiac hypertrophy to have developed. Heart weight, LV weight and their respective indices were significantly greater in the LVH compared to control WKY group as shown in Table 3. An increase in heart and left ventricle weight would suggest an increased exertion of the muscle due to increased activation of β_1 receptors. At the same time, β_2 receptors would also be activated in the peripheral vasculature due to isoprenaline. This would result in vasodilation, which may be a possible reason for the increase in pulse pressure. It could be argued that this vasodilation would decrease after-load resulting in a greater force of contraction by the ventricular muscle to ensure a maintained or even increased blood pressure. This increased exertion of the heart, could go part way to explaining the increased thickness of the myocardium and reduced internal diameter of the left ventricle of the LVH group as shown in Table 3 and Figure 2.

The kidney index was higher in the LVH group and the accompanying hypokalemia may be considered as a factor contributing to the renal hypertrophy (36). Plasma levels of angiotensin are increased in LVH, which may also play role in renal hypertrophy (37).

This increase in cardiac mass may result from a breakdown in the meshwork of elastin collagen membrane (ECM) in the heart, which results in structural and geometrical changes in the left ventricle as shown in Figure 4. All these changes are caused by unsymmetrical, unorganized arrangements of myofibers and breakdown of elastin and increased collagen deposition. The arrows in Figure 4A showed symmetrical and organized arrangement of myofibers while characteristic striated cardiac muscle and normal cardiac nuclei. Figure 4B showed necrotic tissues, and presence of fibroblast confirms the fibrosis in this model of LVH. These changes are indicative of changes in elastin to collagen ratio in cardiac muscle. This turnover in elastin to collagen ratio needs to be explored by evaluating the changes in activity of different matrix metalloproteinases (MMPs).

A few studies have investigated the myotoxic effects of β adrenergic receptors especially β_2 receptors in soleus muscles (38). This myotoxic effect of isoprenaline is likely to be one of the reasons for the weight loss in LVH group as compared to WKY. Moreover, chronic administrations of β adrenergic agonists are noted for causing loss of both body weight and fat (39). Thus, chronic administration of isoprenaline and raised levels of noradrenaline due

to the caffeine together will contribute to the reduced body weight in the LVH group of rats. Another contributory factor for the blunted weight gain in the LVH group may be due to lower water intake and greater urine output in these animals. This may simply reflect energy turnover in the LVH group. These findings are in line with previous findings (3, 4).

Renal function parameters were studied to determine the status of renal functional capacity. It is well known that sympathetic nerves richly innervate the kidneys (40) and influence renal hemodynamic and fluid reabsorption to ensure both long and short term regulation of extracellular fluid volume and arterial blood pressure (41, 42). In LVH, β_1 receptor activity is enhanced due to isoprenaline and caffeine which may impact on renal function. Creatinine clearance, which is a measure of glomerular filteration rate (GFR), was not significantly different in either the LVH or control groups indicating a satisfactory filtration capacity of the kidney. Furthermore, the elevated levels of FENa and FEK indicate decreased reabsorption of these electrolytes from proximal and distal tubules suggesting an early derangement of tubular reabsorption and excretion. One possible reason may be due to the involvement of α adrenergic receptors in the kidneys which play a major role in tubular Na and water reabsorption (43). Secondly, hypernatremia is associated with caffeine administration (44).These findings of elevated fractional excretions of sodium and potassium are consistent with changes produced by caffeine in the body (45). Hypokalemia, as reported in the present study, usually results from damaged proximal tubules as a result of increased ammoniagenesis (36). Urine flow rate was higher in the LVH group as compared to the control WKY group. This increase in urine flow rate and increased urine output in the LVH group may be due to a diuretic action of caffeine, which has been widely reported (28, 45-48).

The hypokalemia observed in the LVH group was associated with the development of LVH and has been shown to be involved with stimulating myocardial growth (48). The decreased U_{Na}/U_K ratio in the LVH group would be consistent with an elevated aldosterone secretion, which would act on the collecting tubules to cause hyper-excretion of potassium. Renal cortical blood perfusion (RCBP) was decreased about 30% in the LVH which would indicate a compromised blood perfusion through kidney. Despite a normal creatinine clearance in both control WKY and LVH WKY, the RCBP was decreased in the LVH WKY group but the underlying mechanism remains unclear. One of the possibility is due to increased regional concentration of catecholamines, especially noradrenaline, causing increased vasoconstriction leading to reduced RCBP. Increased LV mass is proportional to increased sympathetic nerve activity (49). This increased sympathetic nerve activity may cause renal sympathetic nerve to release more nordrenaline in local region thus causing increased vasoconstriction and reduced RCBP. That is fact because free redicals which are elevated in this model are involved in sympathetic neurotransmission to enhance noradrenaline production by increasing the calcium influx into nerve terminals (50).

Arterial stiffness, along with SBP, is marker of CV diseases which will elevate after load and can impact on coronary perfusion (51, 52). Arterial stiffness, as reflected by the pulse wave velocity, is considered to be an indicator of endothelial dysfunction (53). Arterial stiffness is referred to many pathological condition including altered anticoagulant and anti-inflammatory properties of endothelium, impaired modulation of vascular growth and dysregulation of vascular remodelling (54). An ample data considered the arterial stiffness as loss of vasorelaxation ability of blood vessels due to loss of NO. In present model of LVH, plasma levels of LVH WKY is reduced that may lead to arterial dysfunction. This reduced levels may be due to decreased expression of endothelial nitric oxide synthase (eNOS) (55), lack of substrate of eNOS, (56) or accelerated degradation of NO by reactive oxygen species (ROS) (57). In the present study, pulse wave velocity was significantly higher (33%) in the LVH group indicating a higher arterial stiffness as compared to the control WKY. Interestingly, vasoconstriction and elevated blood pressure is usually associated with greater arterial stiffness, which is a predictor of cardiovascular diseases (51). Although isoprenaline produces a peripheral vasodilation, this action may be counteracted by an increased noradrenaline production induced by caffeine administration. These elevated levels of noradrenaline and caffeine could cause an overall vasoconstriction and make the vasculature stiffer. Thus, the overall net effect is an increased left ventricular after-load and greater arterial stiffness ultimately leading to endothelial dysfunction.

Oxidative stress play an imperative role in cardiac and vascular abnormalities in different types of cardiovascular diseases that is why any antioxidant therapy may be beneficial for combating against these diseases (58). So, elevated level of MDA in plasma, which is enzymatic marker of oxidative

stress while attenuated level of SOD which is enzymatic marker of antioxidant mechanism, indicate of oxidative stress in LVH. This shows imbalance between prooxidants and antioxidant in LVH. Other antioxidant parameters like GSH and T-AOC is also attenuated indicating dominant role of prooxidant mechanism in LVH. The levels of NO in plasma is also reduced in LVH WKY group due to involvement of ROS (57).

There are relatively few good invasive models of LVH which produce consistent results with low mortality. One approach in the rat has been to ligate the coronary artery (59) but this has high mortality even in the hands of experienced investigators. An alternative is banding of the descending abdominal aorta (60), which results in an increase after-load but requires extensive surgery. More recently, a non-invasive vascular targeted approach using external lasers has been developed, which may have great potential (61, 62) but is dependent on extensive support in terms of staff and equipment. This has resulted in the drive to develop non-invasive models using pharmacological approaches and has included the use of compounds like caffeine and isoprenaline alone and in combination (4). There are relatively few limitations with the non-invasive models and a consistent level of cardiac dysfunction is achieved. The present model was developed and modified to achieve both objectives. This model has also shown reduced reabsorption capacity and increased excretion ability of kidney. Moreover, oxidative stress and arterial stiffness is increased while renal cortical blood perfusion is decreased in this model of LVH.

Acknowledgments

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GENERAL

OPINIONS AND ATTITUDES OF CZECH CITIZENS ON SELECTED ISSUES OF DRUGS IN SOCIETY

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Abstract: The social sciences within health care and pharmacy provide various points of view on issues often managed by professionals both inside and outside of the health care system. This multidisciplinary perspective often leads to divergent views and sometimes seemingly contradictory viewpoints and solutions. This paper is devoted to the opinions and attitudes of Czech citizens on selected issues of healthcare and pharmacy, the elucidation of which could be a source for understanding the relationships among participants in our pluralist health care system as a whole. This article presents the results of a sociological survey featuring a representative sample in terms of gender, age and region of 1,797 respondents from the Czech population aged 15 and over. The research is focused on the issues of self-medication, choice of pharmacy, out of pocket expenditures on prescription drugs and over-the-counter medicines, experiences with side effects and finally an evaluation of patient comprehension of information leaflets accompanying health care products. According to the results presented here, at the onset of a health problem most of the population tries to treat the condition themselves before seeing a doctor (54.1% always, 30.9% sometimes). Over-the-counter drugs are purchased generally in classic pharmacies without self-service (96.1%). The choice of pharmacy is determined primarily by proximity, with much less importance placed on personal experience or lower price. According to the survey, Czechs spend around 150 CZK (€ 5.9) for over-the-counter medicines and around 143 CZK (€ 5.6) for prescription drugs per month; 77.9% of Czech citizens were shown to understand the information provided on package leaflets. These data help to understand the perception, orientation and behavior of the patient in the healthcare system, with the ultimate goal of leading to higher system effectiveness as well as greater satisfaction for all parties involved.

Keywords: pharmacy; self-medication; drug consumption; drug expenditure; package leaflets; the Czech Republic

In the Czech system, health care is paid for mainly by public health insurance, supplemented by small fees paid by patients. Among European countries the Czech Republic devotes one of the largest shares of its national budget toward funding public health, thus the level of expenditure by the patient (16% of the total) is one of the lowest in the EU (1, 2). The reform measures implemented by the government in 2008 to increase participation have a significant influence on the behavior of Czech patients. The introduction of various types of regulatory fees, however, may bring a range of socio-economic problems, for example increasing the burden of citizens, affecting pensioners and older individuals most drastically (3).

Providing health care is becoming one of the largest sectors of the global economy, with all developed countries recording an effort to improve the health status of their citizens. Not always, however, does the amount of money devoted to health care show corresponding results in the improvement of the overall health of the population as described by international standards (4). Rapid technological change, rising patient expectations and an aging population are driving the need for many reforms in the health sector (1). Unquestionably, the health status of a population, represented for example by average life expectancy, has a relatively strong link to the overall economic level of the country. In comparison with the health values of other European Union countries (EU-27), the Czech Republic remains on the border between average and poorer nations (5).

Despite the decreased spending over the last two decades of reforms and changes which have taken place not only in healthcare but in all sectors,

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the Czech health care system has become measurably more efficient (6, 7). In recent years throughout the Czech health care system, however, reform and partial changes have increasingly influenced public opinion. These developments are often driven not only by economic and organizational need but also by the political background, with which they are closely associated by individual citizen-voters. To insure citizens' maximum understanding of the necessity and effectiveness of every reform, data from public opinion surveys should be carefully analyzed and the results taken into account.

Pharmaceuticals significantly affect society, and the application of appropriate drug policies likewise affect the quality of life in that culture. Citizens themselves can significantly alter the role of specific drugs in terms of prevention as well as causal and symptomatic treatment. Our intention thus is to find, identify and exploit these relationships among regulators, health care professionals and individuals for the benefit of both patients and the society at large. The initial reaction when an individual first experiences a health problem is often a determining factor in effective treatment. People usually try to treat themselves, and only when they fail to achieve results do they see a doctor. Self-medication has increasingly become an important area of healthcare, shifting the responsibility for the treatment of minor ailments to the patient himself (8).

In self-medication the patient usually uses nonprescription medicines, and the entire process of selection and the proper use of substances can be supported significantly by the expert advice provided in pharmacies (9). Despite the fact that online pharmacies must be staffed by a university-educated medical staff which ensures the quality and safety of the medicines marketed, this kind of one-to-one advice to the patient cannot be provided in internet sales. Further, a much more dangerous situation arises from internet transactions outside of regulated pharmacies. These often illegal businesses cannot guarantee the quality or safety of their products, which often come from counterfeiters and may have drastic or even deadly consequences on the health of the buyers.

In legal cases, only authorized pharmacies can offer to sell OTC drugs for the fulfillment of conditions stipulated by law in the Czech Republic. Approved common pharmacy can expand their activities on the mail order dispensing, that offer drug sales through the website. In this way it is possible to offer only registered OTC medicinal products (not prescription-only medicinal products) (10). Another widely frequented point of sale of common medicines are outlets such as drugstores and petrol stations which have a license for the sale of selected medicinal preparations. At any rate employees at these establishments have gone through special training and can ensure the right conditions for the purchase, storage and sale of such products (11).

Selected medicinal products which may, in accordance with the marketing authorization, be sold without a medical prescription outside pharmacies like i.e., herbal products registered as medicinal products (teas), desinfecting and antiseptic preparations, nicotine replacement therapy and some well-known and long used OTC drugs (ibuprofen, paracetamol, activated charcoal, etc.). A complete list is available in the database of registered medicines (10, 11).

Good practice of the vendors of selected medicinal products shall mean a set of rules which ensure that the sale of these products is conducted in compliance with the requirements governing the quality, safety, and efficacy of selected medicinal products and in compliance with their intended use (11).

In the Czech Republic the citizens' choice of pharmacy is not tied to specific medical facilities, thus the provider of pharmaceutical care can be freely changed. There is no obligation to register in a specific pharmacy and thus enjoy the benefits of a long-term relationship with a particular pharmacist. While there are wide disparities among pharmacies regarding the provision of pharmaceutical care (12), the basic responsibilities of all pharmacies are described and regulated through legislation (13). The patient-pharmacist relationship, the demand for vocational counseling, and other various factors influencing selection and patient satisfaction have been to a limited extent analyzed by surveys in the past (14), but these studies have not worked with representative sociological data.

For proper treatment people need comprehensive and accurate information about their medicines as well as information about the risks and benefits of treatment. The lack of information has been identified as a major factor in why patients do not take their medication as prescribed to them or otherwise recommended (15). It is also vital that this information be provided in a form that can readily be understood. The technical or formal language involved with descriptions of adverse effects along with the enumeration and correct standardization of potential problems as well as information on indications, proper dosage and possible interactions with other medicines is for a number of patients incomprehensible. Studies have found that the likelihood of

adverse effects in patients has been greatly overstated (16).

MATERIAL AND METHODS

In the context of our representative sociological survey of citizens of the Czech Republic we first investigated to what extent respondents engaged in self-treatment when health problems occurred. Our research also focused on the preferred method of purchasing over-the-counter medicines and the choice of pharmacy. Special attention was devoted to OTCs and proprietary medicines issued by prescription. Questions were composed with the goal of determining the number of as well as the range of substances used as well as how much money citizens spend on medications within a certain time period. Another aim of the research was to ascertain the views of citizens on the thoroughness of the information on their medicinal products and the clarity of leaflets. Respondents were also asked whether they had noticed any side effects while taking non-prescription medicines and in such a case how they behaved and whether they had taken advantage of additional pharmacy services.

The questionnaire used in survey study contains two groups of questions. The first group, eleven questions, consisted of questions relating to the person the interviewee. Issues as gender, age, place of residence, marital status, number of children, net household income and other household characteristics, education, occupation and religious affiliation. The second group of questions (the number of the twelve) were specific issues related to the topic of sociological survey described below.

The questions were closed-ended and semi-open-ended according to character of questions.

Data collection

The research plan and project was developed in September and October 2011. An examination of the projected methodology took place in early November 2011, at which point preliminary research was used to verify the instruments, with the wording of the questions tested on a sample of 234 respondents (citizens) on 7-11 November 2011. During this period the interviewers were also briefed and coached.

The field survey itself was carried out in the Czech Republic during the period of 21 November to 4 December 2011. The compiling of the questionnaire forms, validation and data entry into the computer was completed by 19 December 2011. Balancing the data, basic mathematical and statistical analysis of the results as well as the final interpretation of the data base had been carried out by 31 December 2011.

The research was conceived as sociological, with the field survey being conducted through controlled interviews of respondents. The final version of the questionnaire form was determined based on the results of preliminary research. Data collection was ensured by 303 professional interviewers working throughout the Czech Republic. Interviewers read the questions exactly as they appeared on the survey questionnaire. In this structured interviews the choice of answers to the questions was fixed (close-ended) in advance and there was also a possibility of semi-open-ended questions in some cases according to the character of these.

The data (answers) were collected by an interviewers personally in households of respondents chosen randomly according to the parameters set out below.

Study population

The data were obtained from a sample size of 1797 individuals randomly selected using quotas. The file is a representative sample of the Czech population aged 15 years and over, with representativeness being derived from a basic set of the population of the Czech Republic at the age of 15 years and older (17).

The composition of the sample can be described in terms of basic demographic variables. In terms of gender the file is composed of 877 (48.8%) men and 920 (51.2%) women, a ratio which corresponds to the Czech population aged 15 years and over; in terms of relative frequency, the ratio from our sample represented a deviation from the basic set of within 0.1%, and compared with the age breakdown of the basic set the deviation in our study does not exceed 0.2%. By geographical representation, the regions from which our respondents were chosen also corresponded with representative population breakdowns of the Czech Republic; compared with the basic set, the maximum deviation of our samples was 0.2%. Thus it can be concluded that our results are representative of the population of the Czech Republic over 15 years of age in terms of gender, age and region.

Other characteristics not monitored for representativeness but collected in the survey include education, marital status, number of children, size of residence, occupation, amount of net monthly family income and standpoint toward religion. When what seemed to be a statistically significant association was found, the study results noted a possible correlation. Nevertheless, due to the fact that these

data were not monitored for representativeness these associations can finally be interpreted only as tendencies.

Response rate

The field survey was conducted by interviewers who questioned a total of 1,996 randomly selected citizens, each of which were approached with a simple request for an interview on the issue of health care; 199 respondents, i.e., 10.0%, simply refused to be interviewed; 1797 respondents, i.e., 90.0%, agreed to be questioned.

An analysis of refusal to participate based on gender indicates that women were more willing to participate in our research than men. The least willing to participate in the project were men and women aged 65 years or over. It was found that willingness to participate in the survey was lower in the oldest age group, while respondents from the youngest age groups were most willing to cooperate. Overall, it can be said that the rate of refusal to participate in the research was low, a result which possibly reflected the special training of interviewers. The preparation focused on ways of making contact and inducing the trust of respondents along with mode of communication in order to encourage participation in the research after the respondents were issued the special instructions.

Regarding reasons for the refusal to participate, the most common one was lack of time (35.4% of those who declined). The second most common excuse given was a general lack of interest in such participation or a disregard for research (29.3%). Another 16.9% of the respondents who said no gave as the reason a mistrust of our particular project and doubts about its efficacy; 9.3% of those who rejected us said the questionnaire was too long and that they considered such studies as ours unnecessary. 4.6% of the respondents justified their refusal by stating that not enough information was presented about the topic being investigated. The remaining 4.5% of respondents who declined gave reasons of health, reasons other than those specified above or no reason at all.

Data analysis

Statistical data processing was done by SASD 1.4.5 (Statistical Analysis of Social Data). First level indicators and a pivot table of selected 2nd degree indicators were processed first. The degree of dependence of the selected characteristics was based on χ^2 distribution as well as on other test criteria applied according to the nature of indicators. On the basis of this analysis alone data interpretation was conducted.

RESULTS

Czech citizens and self-treatment

How Czechs respond initially in the event of a health problem was investigated by means of closed questions such as: “Do you generally attempt to treat yourself before you visit a doctor?” Respondents had three options: “yes”, “no”, and “sometimes”.

The results showed that more than half of the citizens of the Czech Republic (54.1%) attempt self-treatment before going to the doctor, and less than one third (30.9%) do so occasionally. Only 15.0% of Czechs stated that they never first try to treat themselves; they see a doctor straight away whenever a health problem arises.

Table 1. Motivating factors in pharmacy selection for different groups of the Czech population (in percentage).

	Men (n = 877)	Women (n = 920)	Over 65[1] (n = 316)	Lower education[2] (n = 768)	Higher education[3] (n = 1029)	TOTAL (n = 1797)
Personal experience	25.2	32.2	27.2	22.1	33.7	28.7
Nearest pharmacy	45.8	37.0	37.3	44.0	39.3	41.3
Cheaper prices	18.1	24.2	30.4	24.9	18.6	21.3
Another factor	0.4	0.1	0.3	0.3	0.2	0.3
No preference	10.5	6.5	4.8	8.7	8.2	8.4

[1] The eldest citizens of age 65 and over. [2] People with lower schooling (primary, vocational and technical school graduates). [3] Pople with a higher level of education (secondary school and university graduates).

More women (57.8%) attempt to self-medicate than men (50.3%) do ($p < 0.001$). It was also found that individuals with a higher level of education (56.9% of secondary school and university graduates) try to first treat themselves to a greater extent than people with lower schooling (50.5% of primary, vocational and technical school graduates), ($p < 0.01$).

The possibilities of purchasing OTCs

Our research also examined the behavior of citizens regarding the acquisition of non-prescription medicines with the aim of determining preferred point of purchase. The question investigating these behaviors was formulated as a semi-open one with the possibility for respondents to choose more than one answer: "Regarding non-prescription medicines, which of the following purchase options have you used?" Respondents had the option to choose from the following possible responses: "classic pharmacy", "self-service pharmacy", "internet pharmacy", "outside the pharmacy - drugstore, petrol station", "internet, from a non-pharmacy website", and "other – be specific" with a gap provided for written clarification. Because respondents could select multiple answers, the sum of the relative frequencies exceeds 100%.

The results of this question were unambiguous: in the vast majority of cases (96.1%) Czechs buy over-the-counter medicines in traditional pharmacies. Other purchase options were used to a much lesser extent. Self-service pharmacies were identified by 15.5% of the respondents; drugstores and petrol stations were indicated by 9.9%. Online pharmacies accounted for 7.4% of purchases, while 1.6% of the total was accounted for by internet sales from non-pharmacy websites. Other options, e.g., purchases from stores specializing in herbs, shops specializing in dental care, bought from a friend, etc., were indicated at an extremely low rate (0.5%), ($p < 0.05$ for each).

Motivating factors in pharmacy selection

The way Czechs choose pharmacies is governed by a variety of factors and circumstances which were identified in the research through a semi-open question: "What is the most important factor in your selection of pharmacy?" The choices of replies were "personal experience", "nearest pharmacy", "cheaper prices", "another factor" and "no preference". Respondents were able to choose only one answer, which was regarded as the most influential factor in the decision.

In the selection of pharmacy Czech citizens clearly placed the most emphasis on close proximity. More than two-fifths (41.3%) reported that they simply select the nearest pharmacy when they need to buy or order medicines. For 28.7% of the respondents personal experience is most decisive factor; more than one-fifth of the respondents (21.3%) cited lower prices as the motivating factor. Without any preferences in pharmacy selection were 8.5%. Only 0.3% of those questioned indicated another option, with some of their choices specified as opening hours, accessibility to pharmacists, the possibility of paying by credit or debit card, and how the pharmacist advises customers regarding medications. No other factors were reported.

Men indicated significantly more often that they select the nearest pharmacy or they have no preference; women seem to prefer a more personal experience and lower prices than men. The eldest citizens (65 and over) indicated cheaper prices as the deciding factor. Pharmacies with lower prices at a closer location were preferred by those with lower education (primary school, no university); respondents with a higher level of education (secondary school and university) prefer a more personal experience. For detailed results see Table 1 ($p < 0.01$ for each).

Consumption of OTC drugs

Considerable attention was devoted in this study to both the treatment of people with OTC medicines as well as medicines issued by prescription. The aim of the research here was to determine how much money is spent as well as how many and what kind of medicaments are used regularly.

The first aspect of our research in this area focused on the average cost per month that Czechs spend, with the amount being determined by means of a continuous variable (respondents mentioned a specific amount in CZK). Then those questioned were asked to name specifically the over-the-counter medicines which are usually purchased with this monthly expenditure. The first step in the data analysis was the calculation which showed that Czechs spend on average 149.8 CZK (5.9 EUR) per month on OTC drugs (the standard deviation 223.9 CZK or 8.78 EUR). The margin was 2000 CZK (78.4 EUR), indicating that the smallest amount quoted was 0 CZK/EUR, the highest 2000 CZK (78.4 EUR).

Our analysis shows that 29.3% of Czech citizens do not purchase over-the-counter medicines at all; therefore for them no funds are spent in this area. More than half (51.6%) of Czechs spend 200 CZK (7.8 EUR) per month on non-prescription medi-

cines; for 15.3% the amount ranges from 201 to 500 CZK (19.6 EUR) per month. The remaining 3.8% spend an amount exceeding 500 CZK (19.6 EUR) per month. For graphical representation and comparison of differences between expenditures on OTCs and prescription drugs see Chart 1.

Our study also clearly shows that men more often report an amount of 0 CZK/EUR than women and women more often an amount of 200 CZK (7.8 EUR) or higher than men. This finding, however, does not justify the conclusion that men use OTC products less often, but rather reflects the fact that in families women are in most cases more often in charge of shopping. The survey results also indicate a correlation with age. Higher monthly amounts (201-500 CZK, 7.9-19.6 EUR) are spent by respondents in the oldest age group (over 65 years), with 0 CZK/EUR) more likely to indicated by those from younger age groups. This is likely the result of the greater incidence of common health problems in the elderly and the growing need for the use of appropriate medicines, but also in the fact that for young people these products are usually purchased by their parents. For summarized representation of the results see Table 2 ($p < 0.01$).

With regard to OTC drugs, how many of those products are used daily by Czechs was also measured. The average number of OTC drugs used every day is 0.76 (the standard deviation 1.09). In this case the range was from 0 (no OTCs) to 10 (the highest number of OTCs per day indicated).

More than half of Czech citizens (55.1%) indicated that they take no OTC drugs diurnally. Approximately one quarter (25.3%) said that regularly each day they take one OTC drug, another one-fifth of them more than one (12.6% indicated two OTCs daily and 7% three or more OTCs). For graphical representation and comparison of total

Table 2. Total monthly expenditures on OTC drugs by gender and age of the Czech population (in percentage).

	Men (n = 877)	Women (n = 920)	Under 65[1] (n = 1481)	Over 65[2] (n = 316)	TOTAL (n = 1797)
0 CZK/EUR	34.7	24.1	30.3	24.4	29.3
1-200 CZK (up to 7.8 EUR)	49.9	53.3	52.5	47.8	51.6
201-500 CZK (up to 19.6 EUR)	12.7	17.8	13.7	22.8	15.3
501 CZK (19.6 EUR) and more	2.7	4.8	3.5	5.0	3.8

[1] The younger age groups of age under 65 years. [2] The eldest citizens of age 65 and over.

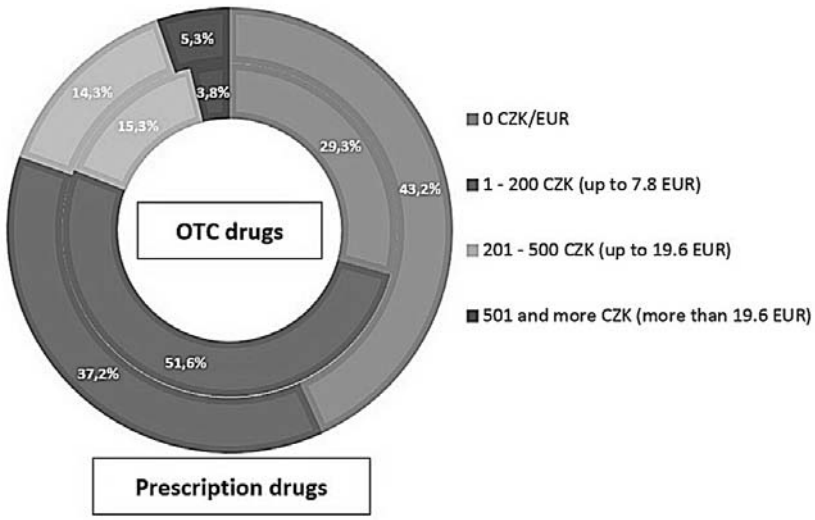

Chart 1. Comparison of total monthly expenditures on prescription drugs (the outer ring) and OTC drugs (the inner ring) of the Czech population aged 15 and over (n = 1797)

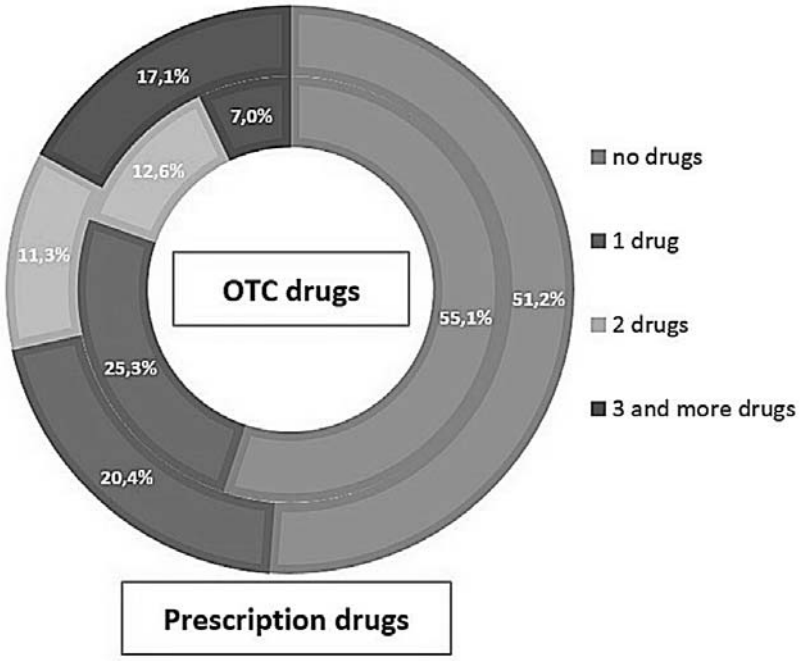

Chart 2. Comparison of total number of daily used prescription drugs (the outer ring) and OTC drugs (the inner ring) of the Czech population aged 15 and over (n = 1797)

number of daily used OTCs and prescription drugs see Chart 2.

Our tests of statistical significance have identified a number of significant relationships ($p < 0.01$) between sociodemographic characteristics and the regular diurnal use of OTC drugs. These products were clearly shown to be used daily more often by women (57% of women daily use one and more OTCs), with significantly more men reporting no use at all (62.4% of men).

A strong linkage ($p < 0.01$) between age and daily use was also found. Based on our analysis it can be concluded that regular diurnal use of OTC drugs increases with age: the younger the respondents, the lower the use. The reason for this is apparently the increasing number of health problems which occur as an individual grows older. In this way age as a factor also operates through marital status. With less products being used by unmarried people (on average 0.54 OTC per day), more by widowed individuals (on average 1.05 OTC per day). A particularly interesting correlation was found between daily use of OTC drugs and religious belief: more OTCs use was reported by Czechs who indicated that they were religious; less was indicated by those with no religious belief. But correlation with age is the reason for this also here, because in the Czech Republic older people in particular have been found to be more religious (18). No real correlation was found between religious beliefs and consumption of OTC drugs, everything is related only to age.

Adverse effects of OTCs

The safe use of (especially new) medicines and an overall deeper relationship between patient and pharmacist is facilitated by the mandatory reporting of unexpected side effects to a central control authority. In the Czech Republic this process is administered by the National Institute for Drug Control.

Within the context of our research into drug use one of the objectives was to determine what experience Czechs have had with side effects of non-prescription medications and how they handle these events. The question relevant to these experiences was worded thusly: "Have you ever noticed side effects after taking OTC drugs?" Respondents could choose one of the following answers: "1 – Yes, I have experienced side effects and I reported them to a pharmacist or physician; 2 – Yes, I have experienced side effects, but I did not report them; 3 – No, I have never noticed side effects."

The results show that almost four-fifths (79.3%) of Czechs reported never having experienced any side effects in connection with OTC med-

icines. Another 11.4% indicated that they had reported adverse effects to a pharmacist or doctor, with the remaining respondents (9.3%) indicating that had experienced adverse reactions, but did not report them.

Significantly more men (83.6%) than women (75.2%) reported they had not experienced side effects from OTC drugs ($p < 0.001$). No other statistically significant links to sociodemographic characteristics were identified.

Consumption of prescription drugs

In the same way that the use of OTC drugs was investigated, prescription drugs were studied in terms of the average monthly expenditure and how many of these products are used regularly on a daily basis. Respondents were to indicate not only a specific amount or quantity, but also to specify which medicinal products are used.

The data analysis shows that Czechs spend on average 143.0 CZK (5.6 EUR) per month on prescription drugs (the standard deviation 283.3 CZK or 11.1 EUR). Financial expenditures indicated in this regard ranged from 0 to 5100 CZK (0 to 200 EUR).

The analysis showed that 43.2% of the respondents stated that they did not allocate any monthly funds toward prescription drugs. Another 37.2% of the respondents indicated that they spent on average of 200 CZK (7.8 EUR) per month on prescription drugs; 14.3% said more than 200 CZK (7.8 EUR) and 5.3% more than 500 CZK (19.6 EUR) monthly. For graphical representation and comparison of differences between expenditures on prescription drugs and OTCs see Chart 1.

As in the case of OTC drugs, the second-stage analyses showed that significantly greater financial expenditures were made by women, specifically between 201 CZK and 500 CZK (7.9 and 19.6 EUR) per month, while significantly more often men reported 0 CZK/EUR. Despite the presence of the phrase "you personally" in the survey question, the fact that women buy more products in total for the entire family may have influenced these results.

Again a strong link to age was found. The result that no funds at all are spent for prescription drugs was reported significantly more often by younger age groups (under 44 years of age). The finding that the amount of monthly financial resources allocated increased with the age of the respondent can likely be linked to the increase of health problems as individuals grow older, as stated above regarding OTCs.

Also as described above, age also operates through marital status and religious belief (18). To a significantly greater extent unmarried respondents indicated no funds spent monthly on prescription drugs; widowed individuals indicated higher expenditures. Similarly, the fact that higher expenditures were described by those claiming to be religious is likely mediated by the fact that in the Czech Republic a greater percentage of these individuals are senior citizens. For summarized representation of the results see Table 3 ($p < 0.01$).

How many prescription drugs are used daily by the average Czech was also investigated. In this case the value is 1.24 (the standard deviation 1.97), indicating that Czechs consume on average more than one prescription drug daily. The range here is from 0-17, i.e., one case was identified in which the respondent reported taking 17 prescription drugs per day.

A bit more than half of the citizens of the Czech Republic (51.2%) stated that at present they take no prescription drug daily. Approximately one-fifth (20.4%) stated they regularly use daily one drug, while the remaining 28.4% indicated two or more products. Combining these latter figures it can be said that about half of Czechs aged 15 or older regularly use a prescription drug at least once a day. For graphical representation and comparison of total number of daily used and prescription drugs OTCs see Chart 2.

Regarding products requiring a prescription as is the case with those that do not, significantly more

Table 3. Total monthly expenditures on prescription drugs by gender and age of the Czech population (in percentage).

	Men (n = 877)	Women (n = 920)	Under 44[1] (n = 871)	Over 44[2] (n = 926)	TOTAL (n = 1797)
0 CZK/EUR	48.8	37.8	55.6	31.5	43.2
1-200 CZK (up to 7.8 EUR)	35.9	38.5	34.8	39.5	37.2
201-500 CZK (up to 19.6 EUR)	10.6	17.8	8.1	20.0	14.3
501 CZK (19.6 EUR) and more	4.7	6.0	1.5	9.0	5.3

[1] The younger age groups of age under 44 years. [2] The eldest citizens of age 44 and over.

Table 4. Understanding of package leaflets for different groups of the Czech population (in percentage).

	Men (n = 877)	Women (n = 920)	Under 30[1] (n = 453)	Over 65[2] (n = 316)	Lower education[3] (n = 768)	Higher education[4] (n = 1029)	TOTAL (n = 1797)
Understand entirely	27.5	37.6	32.5	25.6	24.1	39.1	32.7
Understand almost everything	45.3	45.1	43.5	45.9	46.6	44.1	45.2
Not understand many things	9.9	10.3	8.8	16.2	13.4	7.7	10.1
Not understand at all	0.8	0.5	0.4	2.5	1.2	0.2	0.6
Not read them	16.5	6.5	14.8	9.8	14.7	8.9	11.4

[1] The youngest citizens of age under 30 years. [2] The eldest citizens of age 65 and over. [3] People with lower schooling (primary, vocational and technical school graduates). [4] Pople with a higher level of education (secondary school and university graduates).

women than men reported that they do not use any. Clearly regular daily use of medicines and prescriptions increases with age, with the pivotal point being 44 years of age ($p < 0.01$). Herein the obvious cause of this finding is the growing health problems of the elderly.

Here again age also operates through marital status, with regular daily use being indicated higher by the widowed respondents than unmarried ones. Education is also a factor. A greater number of products used (three or more) was reported at a significantly higher rate by those with only primary, vocational or technical schooling; those with a higher level of education (secondary school, university) were significantly more likely to report that they use no prescription medical product regularly every day (all with $p < 0.01$).

Rating of the clarity of package leaflets

Participants in our study were asked to evaluate the clarity of leaflets enclosed in medicine packaging. They were asked to do so through a closed question with a standardized five-point Likert scale of answers.

Most Czechs (77.9%) reported that they understood the package leaflets in medicinal products in part or in full. Of these, 32.7% said regarding herself / himself "I understand entirely" these leaflets; 45.2% stated "I understand almost everything" the information. The answer "I do not understand many things" was chosen by 10.1% of those questioned, while only 0.6% said "I do not understand at all". The remaining 11.4% of the research participants reported regarding leaflets "I do not know because I do not read them" ($p < 0.001$ for each).

Men significantly more often chose the answer "I do not know because I do not read them"; significantly more women selected "I understand entirely" the leaflets in medical packets.

The youngest age group often chose the answer "I do not know because I do not read them", and the oldest citizens reported more often "I do not understand many things". A lower level of understanding and more answers of "I do not know because I do not read them" was indicated by respondents with lower education, whereas those with a higher level of education stated significantly more often "I understand entirely" package leaflets. For the numerical results of different groups see Table 4 ($p < 0.001$).

CONCLUSIONS

The study showed that self-medication and pharmaceutical care in pharmacies are very important not only for savings in health care financing as a whole, but also for the patients themselves, because the most of them attempt self-treatment before going to the doctor (54.1% always, 30.9% sometimes). The vast majority of the Czech citizens choose classical pharmacy for it and the main motivating factor in pharmacy selection are distance and availability (they prefer the location before price and quality of care provided). 79.3% of Czechs have never had any experience with side effects in connection with the use of OTC drugs. Understanding of package leaflets information by patients is very good, because they said that they understood all (32.7%) or almost everything (45.2%) of the information.

Czechs spend on average 149.8 CZK (5.9 EUR) per month on OTC drugs and likewise on prescription drugs – 143.0 CZK (5.6 EUR) per month and it is still one of the lowest participation (out-of-pocket expenditures) among OECD countries.

DISCUSSION

The optimization of drug therapy has traditionally involved a physician, and in the concept of clinical pharmacy also a pharmacist. The idea of pharmaceutical care in keeping with the decision-making on drug therapy includes also the patient and the drug therapy management also payer of the provided health care (health insurance company). Drug therapy management can be considered as a strategy to incorporate the philosophy of pharmaceutical care into everyday practice of pharmacies (19).

The Czech Republic ranks among the countries with the lowest levels of private expenditure (20) on health care and this situation has not changed significantly even after the introduction of nominal regulatory fees (21). The burden of Czech households increased from 2.15% of their net income to 2.63% in 2008 and to 2.55% in 2009 after the implementation of user fees (3). The increasing private spending on drugs in recent years has not been so significant as in some neighboring countries. For example the private expenditures on drugs were financial burden for the Polish population in recent years. In 2000, 14% of the Polish households spent more than 10% of their income on medications and the share increased over the decade to reach 18% in 2009 (22).

However, Czech citizens perceive their private health expenditure as being much higher than it is in reality. While in the law covering the Czech public health insurance program it is provided that in every group of medicines at least one medicinal product is paid for in full by the health insurance, this applies to only certain groups of active substances provided for in an annex of this law (23, 24). Often Czechs have to pay surcharge for medicines, with the average value in the survey being reported at 143 CZK (5.6 EUR) per month. In official statistical data this average surcharge was cited as half that amount – 72 CZK (2.8 EUR) (2). This difference, in perception by citizens of higher personal expenditure than is really the case, can be seen as an important sociological parameter. Raising the awareness of Czechs about their actual expenditure on drugs as opposed to what they think, could be considered a major contribution of our survey. Along with other considerations, the feeling that one is spending more than is actually the case is also exacerbated in many chronic patients, for example with those who must purchase a selection of concomitant medications every three months. In these cases this accumulated amount could distort the picture of the actual monthly cost.

OECD Health Data are a good source for detailed information about health expenditures. It is good for understanding of health policy and comparison providing of health care in different countries. However, any available official estimates of private expenditure (and their publishing in OECD Health Data) have proven not to be reliable according to some studies (25). Therefore, the sociological surveys and other different types of studies are so important for better understanding and comparison of national diversity from many points of view.

The result of the survey that more than half of Czechs attempt to treat health problems themselves before seeing a doctor should be seen as an important motivating factor for improving the operation of pharmacies, which serve as an ideal place for preliminary consultation. The potential counseling and assistance from pharmacists regarding self-medication, thus only sending the patient to a doctor in the case of a serious problem, brings additional cost savings to the healthcare system. The two regulatory fees, one for doctor visits and another for prescriptions, have certainly enhanced pharmaceutical care in terms of self-treatment as well as increased savings by almost 22% due to an overall decrease in the number of items issued per prescription (26).

According to the another sociological study, the main source of information about medicines are for the Czech citizens the package leaflets (58.2%), the prescribing physician (58.2%) and only 37.0% of them obtain information from their pharmacist (27). According to the study conducted one year after the implementation of regulatory fees, a proportional distribution of the results is the same (relative frequency; annual change): the package leaflets (67.7%; +9.5%), the prescibing physician (56.3%; -1.9%) and pharmacist (44.0%; +7.0%) (28). Because respondents could select multiple answers, the sum of the relative frequencies exceeds 100%. The clarity of package leaflets is an important and reasoned subject of interest. Participants in our study understood the package leaflets in medicinal products in part or in full. Men did not read them more often and significantly more women understood them entirely. In another study, the Czech citizens before taking a new drug in 80% of cases read leaflets and follow recommendations of them (more women – 86% than men – 75%) (29).

Regarding self-treatment and the opportunity to purchase medicines, the vast majority of Czechs choose classic pharmacies in which the pharmacist can provide technical assistance and other recommendations. Neither personal experience (29%) nor price (21%) was found to be the leading deciding factor in the selection of pharmacy; proximity affected the choice most often (41%), with the nearest available facility being chosen. This results (proximity and affordable of pharmacies) is related to the opinions of the Czech citizens from other studies (29); in case of legal dispensing of some drugs outside classical pharmacies (and in case of cheaper price and better accessibility of dispensing place), 41% of respondents would use such a place (29). The same view like a currently legislation (10) has 59% of Czech citizens who disagree with sales outside pharmacies (29). The most preferred alternative places of this sales are drugstores (26% of whole population) and supermarkets (25% of whole population) (29).

The finding that religious individuals regularly use more medicines daily than non-religious people could lead to erroneous conclusions: this factor is closely related to age, as in the Czech Republic many more individuals in the age group above 70 years identify themselves as religious (68%), a statistic which represents a much higher average than that of the entire population 15 years and older (39% of which are religious) (18). There is no correlation between religious beliefs and consumption of drugs. According to the demographic characteristics of the Czech population, consumption of drugs s not related to religious beliefs but only to age.

Data from the present project can serve not only to facilitate wider understanding and possibly suggest potential nationwide changes and major decisions, but also help shape the smaller sub-goals of regional institutions, private facilities and future research projects. Nationwide changes over the last two decades including but not limited to reforms in the field of Czech medical care can be re-evaluated and developed further thanks to the type of representative sociological survey that was carried out in this project (30). This kind of sociodemographic information about personal attitudes, beliefs and habits is of vital importance to health providers (doctors and pharmacists) who are in daily contact with the end user (patient). Thus medical and pharmaceutical professionals may focus their efforts on the goal of increased efficiency and the greater satisfaction of all involved in the health care process.

For a better understanding and comparing, the values in Czech crowns (CZK) were converted into Euro (EUR) at the average exchange rate values during data collection, which was 25.5 CZK/EUR (31).

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PHYSICIANS' VALUATION OF INDIVIDUAL MEDICATION MANAGEMENT SYSTEM (IMMS) IMPLEMENTATION IN POZNAŃ (POLAND) COMMUNITY PHARMACIES

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Abstract: The reason of growing Pharmaceutical Care (PC) popularity in Poland and all over the world in recent years is connected with a new idea which separates pharmacists from a stereotype of drug store, dealing only with issuing medicines in adequate doses. There is an intention of emphasizing their qualifications and maximizing the use of them. One of the essential part of PC is preparing Individual Medication Management System (IMMS) which may provide individualized pharmacotherapy for patient. The aim of the study was to assess the physicians' opinion about implementation of IMMS in Polish community pharmacies and to evaluate physician-pharmacist cooperation. A cross sectional study was carried out from April 2013 to December 2013 by a pharmacist (authors' of the study). The survey covered 103 physicians (35.9% men and 64.1% women) providing medical services in Poznań. The respondents obtained an anonymous questionnaire with a brief information about IMMS. The results of the study confirmed that 90.3% of physicians would recommend IMMS to their patients. They believed that 72.8% of the patients would be interested in this service. According to 74.8% of doctors, especially with a specialization in cardiology, family medicine, and without specialty, IMMS might contribute to the PC development ($p < 0.0001$). The respondents (56.3%) confirmed their collaboration with at least 1 pharmacist and 79.6% declared the possibility of the cooperation by using IMMS. This study provides new data about implementation of IMMS in Poland. This innovatory service could be the chance both for patient and physicians to increase the safety and effectiveness of pharmacotherapy and for pharmacists who are intended to highlight their role as a part of health care system. The physicians' positive opinion provide the opportunity to implement IMMS in Polish community pharmacies.

Keywords: Individual Medication Management System, pharmaceutical care, pharmacist, physician

The pharmaceutical care (PC) popularity is growing permanently mainly because of the intention to change the perception of pharmacist's profession (1, 2). This new service is the direct, responsible provision of medication-related care for the purpose of achieving definite outcomes that improves a patient's quality of life (3). Increasing patient's health awareness and unlimited access to pharmacists were mostly the reason to start popularizing and implementing PC also in Poland. This situation requires from pharmacist cooperation with patients, physicians and other health care professions effectively to avoid e.g., patient's nonadherence, which is determined as the number of doses not taken or taken incorrectly that jeopardizes the patient's therapeutic outcome (4). Many studies confirm that mostly people with chronic diseases don't follow up the physician guidelines, which generate increased mortality rate (5).

Individual Medication Management System (IMMS) is one of the proposals to resolve this problem. It is an example of a disposable, blister pack system for dosing solid drugs which is divided into special compartments intended for specific time of

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Table 1. Physicians' frequency distribution of the study.

	Frequency (n)	**Percentage (%)**
Age (years)		
Under 35	21	20.4
35-64	68	66.0
65+	14	13.6
Total	103	100.0
Scientific/Professional degree		
MD	95	92.2
PhD	7	6.8
Prof.	1	1.0
Total	103	100.0
Length of service as a physician		
Under 5 years	15	14.6
5-20 years	33	32.0
Over 20 years	55	53.4
Total	103	100.0
Gender/sex		
Male	37	35.9
Female	66	64.1
Total	103	100.0
Specialty		
Family medicine	12	11.7
Diabetology	13	12.6
Cardiology	17	16.5
Others:	44	42.7
Pediatrics	7	6.8
General surgery	6	5.8
Ophthalmology	6	5.8
Obstetrics and gynecology	6	5.8
Dermatology and venereology	5	4.9
Orthopedics and traumatology	5	4.9
Otolaryngology	5	4.9
Urology	4	3.8
No specialty	17	16.5
Total	103	100.0

the day, on particular days of the week. This has a positive effect on dosing scheme and it helps to eliminate the risk of errors. IMMS is prepared by a pharmacist who is obliged to verify dosing scheme propriety, check relevant doses and eliminate possible drugs interactions. Moreover, a pharmacist is obliged to attach information about the way of taking prepared drugs, possible adverse drug reactions (ADR), and the course of action in case of its occurrence. IMMS is mostly directed for patients with multiple-drug treatment, old aged people, and patients who are treated by many physicians (6).

It is confirmed that IMMS improves patient's adherence. The best results are generated when

health care practitioners cooperate with each other. Completed patient's documentation, gathered information about the disease and taken drugs are the best proposition to resolve pharmacotherapy problems. Besides, different views of specialists are very valuable to obtain the best patient's drug therapy (7, 8). The limitation in practical application concerns patient who intentionally ignores prescribed pharmacotherapy. It may reduce nonadherence but very often professional psychological advice is needed first. IMMS is intended for solid and oral drugs not for effervescent or sublingual tablets. There is also potential possibility of interaction between drug and blister (9, 10).

The aim of the study was to assess the physicians' opinion about implementation of IMMS as an important part of PC in Polish community pharmacies and to evaluate physician-pharmacist cooperation. The study defined also whether IMMS could improve doctors' and pharmacists' cooperation to get proper pharmacotherapy. The data assumed analysis for gender, specialty, scientific/professional degree and length of service as a physician.

MATERIAL AND METHOD

The survey as a cross sectional study was carried out from April 2013 to December 2013 by a pharmacist (authors' of the study). It covered 103 physicians (35.9% men and 64.1% women) providing medical services in Poznań. Each anonymous questionnaire was supplied with a short information brochure about IMMS and was filled in by the physician. The most numerous group of respondents consisted of physicians aged from 35 to 64 years old (66.0%) and with over 20 years length of service as a physician (53.4%). The other age groups were: under 35 – 20.4%, 65 years and more – 13.6%. In terms of the length of service as a physician the remaining groups were: under 5 years – 14.6%, 5–20 years – 32.0%. The majority of the responding participants had no scientific degree (92.2%) and 16.5% of the physicians did not have specialty. Among the respondents 16.5% were cardiologists, 11.7% of them were family medicine doctors and 12.6% were diabetologists. The rest of the specializations were joined together as a group named "physicians of other specialties". Socio-economic data included information about age, sex, specialty, professional/scientific degree and length of service of physician and are presented in Table 1. The study was approved by the ethics review board of Poznan University of Medical Sciences.

The results were statistically analyzed with the use of Statistica 10.0 application (StatSoft®). The relationship between analyzed nominal data was performed by χ^2 test of independence. In case when observed frequencies were low or zero the Fisher-Freeman-Halton exact test was used. All statistical analyses were performed at $p < 0.05$.

RESULTS

The study confirmed that 39.8% questioned, mainly the physicians who specialize in family medicine, ordered their patients the classic dispensers for solid drugs during their everyday practice (Table 2; $p = 0.0353$) and 90.3% of them would recommend the use of IMMS to patients in the future (Fig. 1) They believed that 72.8% of the patients would be interest-

Table. 2. Physicians' opinion concerning recommending drug dispensers to their patients and about IMMS contribution to PC development and their specialty.

	Specialty						
	Family medicine	**Diabetology**	**Cardiology**	**Others**	**No specialty**	**Total**	**p-value**
Recommending classic dispensers for patients							
yes n (%)	8(66.7)	4(30.8)	3(17.6)	6(36.4)	10(58.8)	41(39.8)	0.0353*
no n (%)	4(33.3)	9(60.2)	14(82.4)	28(63.6)	7(41.2)	62(60.2)	
IMMS contributing to PC development							
yes n (%)	10(83.3)	4(30.8)	12(70.6)	35(79.5)	16(94.1)	77(74.8)	
no n (%)	0(0.0)	1(7.7)	0(0.0)	5(11.4)	0(0.0)	6(5.8)	< 0.0001*
no opinion n (%)	2(16.7)	8(61.5)	5(29.4)	4(9.1)	1(5.9)	20(19.4)	

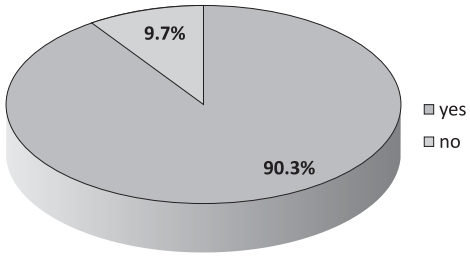

Figure 1. Physicians' opinion concerning the possibility of recommending the use of IMMS to patients, n = 103

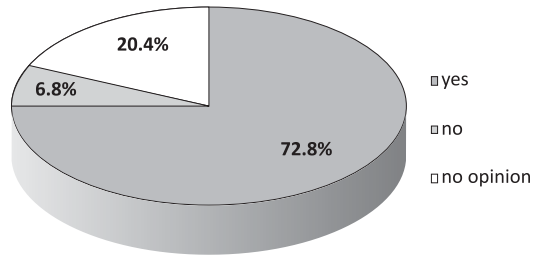

Figure 2. Physicians' opinion concerning patients' interest in using IMMS, n = 103

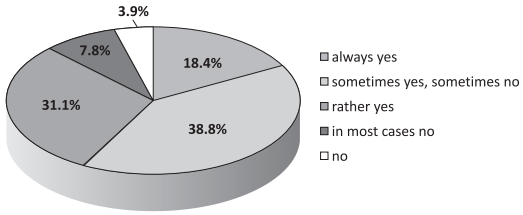

Figure 3. Physicians' opinion about patients' willingness to pay for IMMS, n = 103

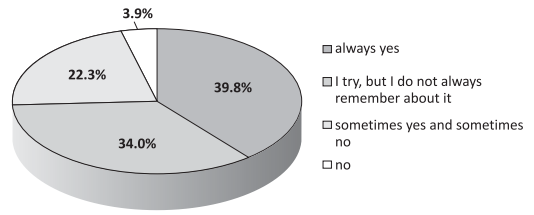

Figure 4. Physicians' opinion concerning paying for IMMS, n = 103

ed in this service (Fig. 2) and that 35.0% of them would pay for it (Fig. 3). The doctors were of opinion that this service should be refunded (43.7%), or paid (37.9%) by the patients (Fig. 4). According to 74.8% of doctors, especially with a specialization in cardiology, family medicine, and without specialty, IMMS might contribute to the development of the PC ($p < 0.0001$). The results are presented in Table 2.

The respondents (56.3%) assessed their collaboration with at least 1 pharmacist (Fig. 5), 79.6% of the participants noticed the possibility of cooperation between physician and pharmacist in order to improve the effectiveness and safety of patient's pharmacotherapy by IMMS (Fig. 6).

Unfortunately, only 18.4% of the respondents indicated that they always have enough time to educate patients about their diseases properly. The physicians (38.8%) responded that sometimes they have enough time to give proper information to the patient. Some doctors (7.8%) states that in most cases they didn't educate their patient's (Fig. 7). Only 39.8% of the physicians claimed that, while prescribing drugs they took into consideration all the medications taken by a patient (Fig. 8). What is more, 70.9% of the physicians noticed the problem of not obeying doctor's recommendations regarding the rules of taking drugs (Fig. 9).

Additional gender, specialty, scientific/professional degree analysis didn't achieve the level of statistical significance.

DISCUSSION AND CONCLUSION

The study confirmed popularity of classic dispensers among physicians and great interest in IMMS. In the opinion of the physicians, this system will be valuable for many patients. In many studies, it is proved that IMMS is better than classic dispenser because it is prepared by the specialist. Pharmacist's knowledge and experience lead to decreasing errors which can occur when patient prepare drug scheme alone. Moreover, pharmacist analyzes patient's pharmacotherapy and can resolve drug problems during providing pharmaceutical care. It is verified that IMMS eliminates polypragmasy, provides proper doses of the drugs and indicates adverse drug reactions (11-13).

According to physicians opinion, IMMS should be refunded or paid by the patient. This new service is a valuable idea for government, insurers and health care team, because of its economical consideration. There are many studies which assess that patient's nonadherence leads to: extension of the length or failure of treatment, more severe course of a disease, the necessity of hospitalization, or even death. In the United States of America it is explored that these are very high valuation, precisely 2.5% of national health funds. Unfortunately, in Poland it is even much higher, precisely 10% of National Health Fund budget (5, 15, 16).

So, it is very important to get a proper cooperation between physician and pharmacist. In this

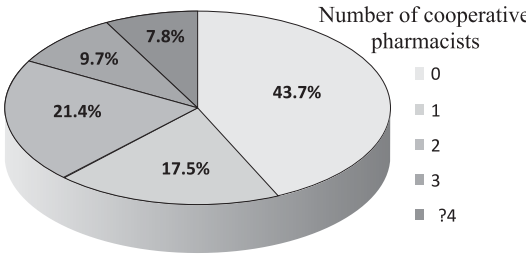

Figure 5. Physicians' opinion concerning cooperation with pharmacists, n = 103

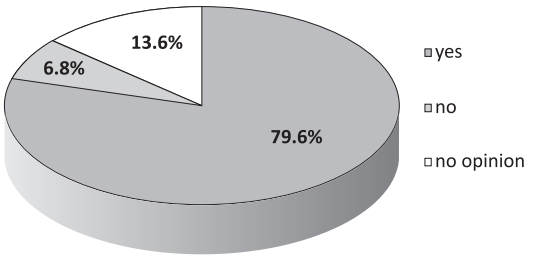

Figure 6. Physicians' opinion concerning partnership with pharmacists' widen by IMMS application, n = 103

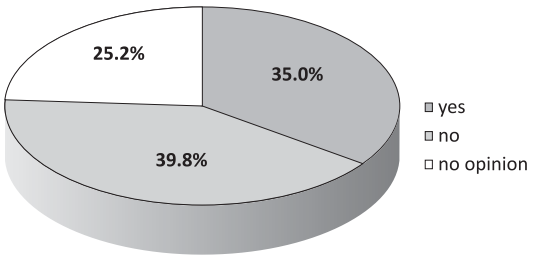

Figure 7. Physicians' opinion concerning having time to educate patients, n = 103

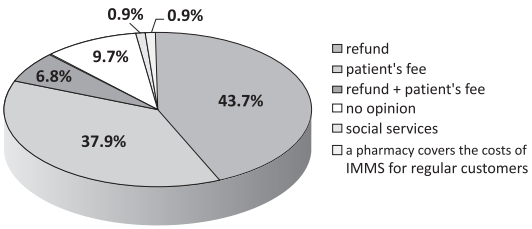

Figure 8. Physicians' opinion concerning taking into consideration all of the medications being taken by patients while prescribing drugs, n = 103

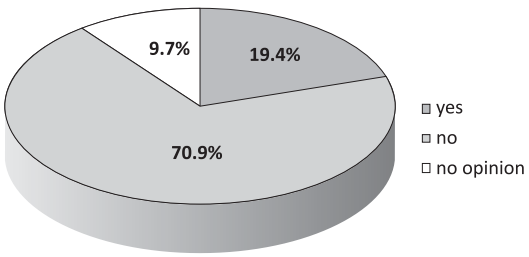

Figure 9. Physicians' opinion concerning patients' following doctors' recommendations, connected with instructions about the rules of taking drugs, n = 103

study, it turns out that about half of the responding physicians were cooperating with pharmacist. In the study from 2012, 35.2% of pharmacists were collaborating with the physicians (16). In many countries, where participants of health care team work together and share experiences from different specialties, it brings a lot of advantages for patients' pharmacotherapy (17-19). It is also very important, especially in the situation when the physician very often do not have enough time for the patient's education. It can lead to many complications connected with e.g., wrong drug application or improper dosage. Many studies describe poor patients education, which should be improved because of its importance in specific pharmacotherapy scheme, especially in chronic diseases (20, 21). IMMS could be the opportunity both for physicians and pharmacist to provide adequate patients' pharmacotherapy. It can have also a positive influence on the improvement of pharmacist-physician partnership. The growing interest in cooperation can lead to more effective informations about patients' health condition and their pharmacotherapy. As a result, it would have an impact on the safety of therapy.

Unfortunately, only some of the physicians take always into consideration all of the medications being taken by patients, while prescribing drugs. This is the factor which increases the opportunity of drugs interaction occurrence, also with OTC medicines. Moreover, it can lead to a situation, when a patient takes more than one drug that magnifies the same side effects or overdose medicine by taking more than one preparation with similar properties. Very often, patient takes the same generic and name-brand drug at the same time, so that it is suitable to check the patient's medication e.g., by IMMS service (22, 23).

IMMS is a new idea to achieve a better patients' pharmacotherapy. It gives a lot of advantages not only for the patient but also for physician-pharmacist cooperation which is valuable in pharmacological treatment. As an innovative idea it may

improve patient's compliance in pharmaceutical care process.

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ANALYSIS OF TRENDS IN LIFE EXPECTANCIES AND *PER CAPITA* GROSS DOMESTIC PRODUCT AS WELL AS PHARMACEUTICAL AND NON-PHARMACEUTICAL HEALTHCARE EXPENDITURES

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Abstract: Life expectancy is a common measure of population health. Macro-perspective based on aggregated data makes it possible to approximate the impact of different levels of pharmaceutical expenditure on general population health status and is often used in cross-country comparisons. The aim of the study was to determine whether there are long-run relations between life expectancy, total healthcare expenditures, and pharmaceutical expenditures in OECD countries. Common trends in *per capita* gross domestic products (GDPs) (excluding healthcare expenditures), *per capita* healthcare expenditures (excluding pharmaceutical expenditures), *per capita* pharmaceutical expenditures, and life expectancies of women and men aged 60 and 65 were analyzed across OECD countries. Short-term effect of pharmaceutical expenditure onto life expectancy was also estimated by regressing the deviations of life expectancies from their long-term trends onto the deviations of pharmaceutical and non-pharmaceutical health expenditures, as well as GDP from their trends. The dataset was created on the basis of OECD Health Data for 34 countries and the years 1991-2010. Life expectancy variables were used as proxies for the health outcomes, whereas the pharmaceutical and healthcare expenditures represented drug and healthcare consumption, respectively. In general, both expenditures and life expectancies tended to increase in all of the analyzed countries; however, the growth rates differed across the countries. The analysis of common trends indicated the existence of common long-term trends in life expectancies and *per capita* GDP as well as pharmaceutical and non-pharmaceutical healthcare expenditures. However, there was no evidence that pharmaceutical expenditures provided additional information about the long-term trends in life expectancies beyond that contained in the GDP series. The analysis based on the deviations of variables from their long-term trends allowed concluding that pharmaceutical expenditures significantly influenced life expectancies in the short run. Non-pharmaceutical healthcare expenditures were found to be significant in one out of four models (for life expectancy of women aged 65), while GDPs were found to be insignificant in all four models. The results of the study indicate that there are common long-term trends in life expectancies and *per capita* GDP as well as pharmaceutical and non-pharmaceutical healthcare expenditures. The available data did not reveal any cause-effect relationship. Other factors, for which the systematic data were not available, may have determined the increase in life expectancy in OECD countries. Significant positive short-term relations between pharmaceutical expenditures and life expectancies in OECD countries were found. The significant short-term effect of pharmaceutical expenditures onto life expectancy means that an increase of pharmaceutical expenditures above long-term trends would lead to a temporary increase in life expectancy above its corresponding long-term trend. However, this effect would not persist as pharmaceutical expenditures and life expectancy would converge to levels determined by the long-term trends.

Keywords: statistical data analysis, gross domestic product, life expectancy, health expenditures

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The work reported in this paper was a part of Work Package 3 within the International Research Project on Financing Quality in Healthcare - InterQuality (co-funded by the 7th Framework Programme (FP7) for Research and Technological Development of the European Union). The aim of the project was to validate pharmaceutical benefit financing models to find promising solutions in the area of pharmaceutical care and provide implementation recommendations for policy-makers from European countries.

The first step in the validation of pharmaceutical benefit financing models was to evaluate them from the macro-perspective. Life expectancy variables were used as proxies for the health outcomes, whereas the pharmaceutical and healthcare expenditures represented drug and healthcare consumption, respectively.

Life expectancy is an indicator of how long a person can expect to live on average, given prevailing mortality rates. In the available literature this indicator was a common measure of population health in general, and was often used as a summary measure when comparing different populations. There was a consensus amongst researchers that health expenditures, reflecting the quality of healthcare, may have a positive impact on the population health status and that this impact should be evaluated. Positive and statistically significant relationship between life expectancy (LE) and pharmaceutical expenditure was found in many studies (1-6). It was found that the consumption of newer drugs was associated with significantly lower mortality than the consumption of older drugs and that the use of newer drugs limits all types of nondrug medical expenditure, resulting in a reduction of the total cost of treating a particular condition (7). Studies carried out from micro-perspective (8, 9) provided valuable insights concerning particular drugs, diseases, therapeutic groups, or individual patients. However, these studies did not inform about the overall effect of drug consumption onto the population health status. Therefore, in order to approximate the impact of pharmaceutical expenditure on general population health status, a macro-perspective based on aggregated data was adopted.

The study analyzed common trends in *per capita* GDPs (excluding health expenditures), *per capita* healthcare expenditures (excluding pharmaceutical expenditures), *per capita* pharmaceutical expenditures and life expectancies of women and men aged 60 and 65 across OECD countries. This approach allowed separating long-term and short-term dynamics in life expectancies and health expenditures. The analysis of common trends made it possible to identify possible long-run relations between life expectancy, total healthcare and pharmaceutical expenditures in OECD countries.

MATERIAL AND METHODS

The dataset containing information about *per capita* GDPs, *per capita* healthcare expenditures, *per capita* pharmaceutical expenditures and life expectancies of women and men aged 60 and 65 was built on the basis of OECD Health Data for 34 OECD countries and the period 1991-2010 (10). Natural logarithms of all the variables were used.

To investigate if there are common trends in *per capita* GDPs, *per capita* healthcare expenditures, *per capita* pharmaceutical expenditures and life expectancies, methods described by Bai (11) were employed. This methodology was chosen as the data under investigation were non-stationary. The idea of this approach was to identify the number of common stochastic trends in two panels of non-stationary time series separately and in a panel of concatenated time series. If the number of common trends in the concatenated panel was lower than the sum of common trends extracted from each of the analyzed panels, it implied the existence of long-run relations between the two analyzed indicators in the countries under investigation.

In order to identify common trends in life expectancies, *per capita* pharmaceutical and non-pharmaceutical healthcare expenditures, and *per capita* GDP, the analysis was carried out in a few stages. First, common trends across countries were identified for each of the variables, allowing for the maximal number of common trends equal to five. Second, the common trends were identified in pairs of variables. Finally, life expectancy was added to various types of expenditures in order to identify the total number of trends that is sufficient to describe non-stationary dynamics of all the indicators.

This approach made it possible to identify those variables which have long run relations with life expectancy. However, it should be noted that the existence of long run relations between life expectancy and another variable did not imply causation.

RESULTS

Figures 1 and 2 show the association between pharmaceutical expenditures and life expectancies of males and females aged 60 for selected OECD countries. The graphs illustrate some general tendencies

which could be observed for the whole panel of countries. Six countries participating in the InterQuality project were selected so that the figures were not overcrowded. In general, in all the countries, increase of both expenditures and life expectancies was observed; however, the growth rates differed between countries. For example, the relative increase in pharmaceutical expenditures in Denmark was much lower than in the US, but the relative increase in life expectancies was rather similar.

The analysis of common trends in each of the analyzed variables revealed two common trends for

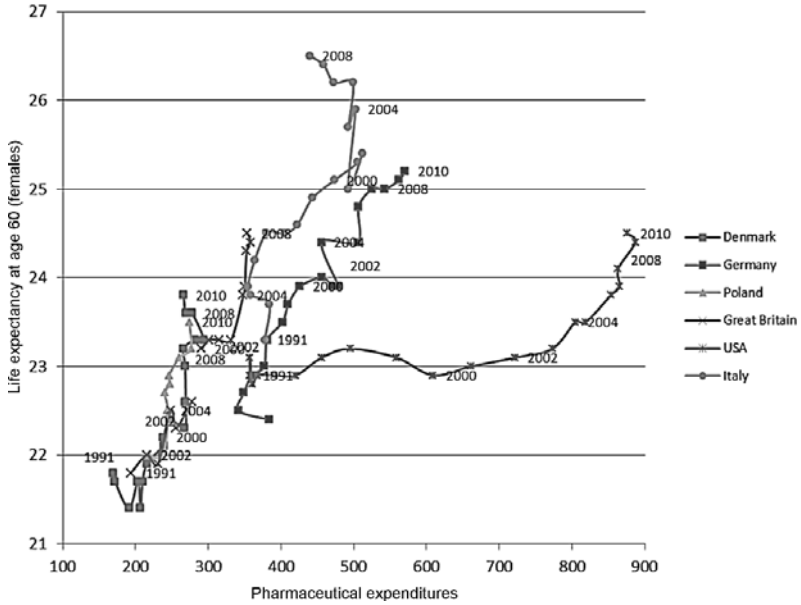

Figure 1. Pharmaceutical expenditures and life expectancy of females aged 60

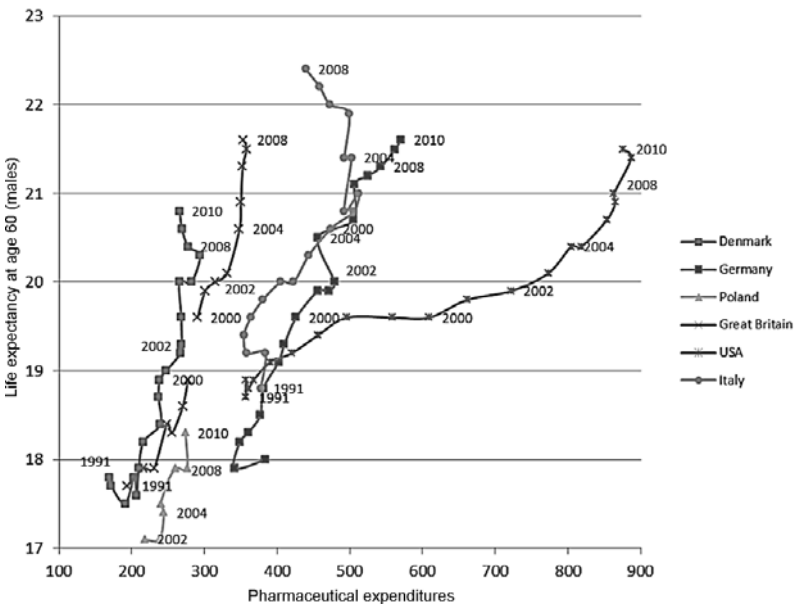

Figure 2. Pharmaceutical expenditures and life expectancy of males aged 60

all four indicators of life expectancy, four common trends for *per capita* GDP, and five common trends for pharmaceutical and non-pharmaceutical healthcare expenditures.

In the second step of the analysis, the number of common trends for pairs of variables was estimated. Combining the data on life expectancies for different ages and genders did not change the estimated number of common trends. It can be suggested that two trends are sufficient to describe common non-stationary dynamics of life expectancies across countries. For any data set combining life expectancies and GDPs, pharmaceutical or non-pharmaceutical healthcare expenditures, there was no increase in the number of non-stationary trends as compared with the maximal number of trends in any of the paired data sets, i.e., common trends in life expectancies can be included in subsets of common trends in GDPs, pharmaceutical or healthcare expenditures. However, the data set of GDPs included two additional common trends and the data sets of pharmaceutical and non-pharmaceutical healthcare expenditures included three additional trends as compared to the data set of life expectancies.

In the last step of the common trends analysis, the number of common trends was estimated for groups of four to seven data sets of indicators. Two common trends were found for the group composed of life expectancies for females and males aged 60 and 65. It means that all considered measures of life expectancies shared the same long-term trends. Five common trends were found for each measure of life expectancy combined with *per capita* pharmaceutical expenditures, *per capita* healthcare expenditures, and *per capita* GDP. Given the results of the analysis conducted for pairs of variables, it can be concluded that there were two common trends that were shared by all the variables in each of these subsets. There were two additional common trends shared by GDP, total healthcare expenditures and pharmaceutical expenditures, and one more trend shared by total healthcare expenditures and pharmaceutical expenditures.

Finally, deviations of life expectancy variables from their long-term trends were analyzed by regressing them on their own lags and onto the lags of deviations of pharmaceutical and non-pharmaceutical healthcare expenditures from their common trends, as well as lags of deviations of GDPs from their trends. The explanatory variables were lagged in order to avoid simultaneity bias. Three-year lags were allowed for each explanatory variable.

The estimation results are presented in Table 1. Significance of lagged dependent variable (at the level of significance equal to 5 percent) for all four indicators of life expectancy indicated that after removing the long-term trends there was large residual time dependence. Deviations of *per capita* GDPs

Table 1. Short-term relationships between de-trended life expectancy variables and expenditure variables.

	*LE*60*F*	*LE*65*F*	*LE*60*M*	*LE*65*M*
PE(-1)	0.024*	0.036	0.041***	0.040**
PE(-2)	0.029*	0.023	-0.011	0.007
PE(-3)	0.028**	0.044**	0.061**	0.063*
HE(-1)	-0.008	0.000	0.014	0.018
HE(-2)	0.019	0.038**	0.005	0.016
HE(-3)	0.015	0.004	-0.001	0.000
GDP(-1)	-0.036	-0.046	-0.080	-0.047
GDP(-2)	-0.062	-0.002	0.100	0.037
GDP(-3)	-0.003	-0.006	-0.026	0.015
dep.var(-1)	0.432***	0.447**	0.414***	0.379***
dep.var(-2)	0.085	0.165***	0.035	0.068*
dep.var(-3)	-0.143***	-0.238***	-0.189***	-0.145***

Notes: All variables represent deviations from the long-term trends. Values in parentheses indicate the lag used and *dep.var.* stands for the dependent variable. Abbreviations: *LE*60*F*, *LE*65*F*, *LE*60*M*, *LE*65*M* stand for life expectancies of females aged 60 and 65 and males aged 60 and 65, respectively. *PE* = pharmaceutical expenditures, *HE* = healthcare expenditures and *GDP* = gross domestic product. *, ** and *** indicate significance at 10%, 5% and 1% levels, respectively.

from long-term trends were found to be insignificant in all four models. This can be interpreted as follows: *per capita* GDP determined long-term trends in life expectancy, but deviations of GDP from the long-term trends had no significant short-term effect on life expectancy.

Deviations of pharmaceutical expenditures from the long-term trends were found to be significant in all four estimated models: short-term expansions of pharmaceutical expenditures had a significant short-term effect onto life expectancy of people aged 60 and 65 (Table 1). Thus, if pharmaceutical expenditure in a country is above its long-term trend in a given year (i.e., there is an unexpected increase in the pharmaceutical expenditure), life expectancy will also be above its long-term trend in a few years following the increase in pharmaceutical expenditure. Non-pharmaceutical healthcare expenditures were found to be significant in one model only (for life expectancy of women aged 65). The lack of a significant partial correlation between life expectancies and non-pharmaceutical healthcare expenditures can be explained by the fact that non-pharmaceutical healthcare expenditures include both current expenditures of the healthcare systems and long-term investment. The long-term investment may have no immediate effect onto health status.

DISCUSSION

The aim of the study was to analyze relations between pharmaceutical expenditures and life expectancy. The analysis used panel data to evaluate common trends in pharmaceutical expenditures and life expectancies across countries. There were two key findings. Firstly, the study revealed significant positive short-term relations between pharmaceutical expenditures and life expectancies in OECD countries. Secondly, it demonstrated that there are common long-term trends in life expectancies and *per capita* GDP as well as pharmaceutical and non-pharmaceutical healthcare expenditures. However, there was no evidence that pharmaceutical expenditures provide additional information about the long-term trends in life expectancies beyond that contained in the GDP series. As the GDP series are correlated with various determinants of life expectancies, which include healthcare expenditures as well as life-style and environmental factors, the common long-term trends in life expectancies can be interpreted as generated by a variety of factors. No specific contribution of healthcare expenditures to these long-term trends can be identified on the basis of the data set under study.

The methodological differences do not allow direct comparisons of the results presented in this paper with other published studies. Nevertheless, the reported conclusions are consistent with other research. In similarity to our analysis, Caliskan et al. (1) found a positive relationship between pharmaceutical spending and life expectancy. The authors used unbalanced panel data of 21 OECD countries over the period from 1985 to 2002. The empirical results showed that pharmaceutical expenditure, measured by *per capita* drug spending, had positive, but heterogeneous effects on life expectancies for females and males of various ages.

A positive effect of drug consumption, measured as *per capita* pharmaceutical expenditure, on population life expectancy at various ages was also confirmed in the study by Shaw et al. (2). They found that pharmaceutical consumption had a positive effect on life expectancies at middle and advanced ages. It was concluded that doubling annual drug spending added about one year of LE for males at the age of 40 and slightly less than a year of LE for females at the age of 65. The estimation was based on cross section data collected for 19 OECD countries.

The link between pharmaceutical expenditures and health outcomes in 14 countries using OECD panel data from the years 1985 to 2001 was also studied by Liu et al. (3). It was confirmed that significant, but marginal gains in population health outcomes were associated with increased pharmaceutical expenditures. A 10% increase in drug spending was connected with a 0.3% increase in female LE at the age of 65. A similar increase was associated with a 0.4% increase in male LE at the age of 65 years and a 0.5% increase at the age of 80 years.

Similar studies were conducted by Crémieux et al. (4, 5), who assessed the statistical relationship between pharmaceutical expenditures and population health outcomes in Canada. The results of the study showed a strong statistical relationship between drug spending and LE at 65 and LE at birth. This link was stronger for private drug expenditure than for public drug expenditure.

The empirical analysis presented in this paper is not free from limitations. Firstly, the choice of adopted methodology limits the conclusions. It does not allow estimating a cause-effect relationship. Existence of common trends does not necessarily mean that systematic increase in pharmaceutical expenditures would significantly increase life expectancy in the long run. The presence of common trends does not exclude heterogeneity in pharmaceutical expenditures and life expectancies across

countries either. As more trends are needed to explain the long-term tendencies in pharmaceutical expenditures than long-term tendencies in life expectancy across the data panel, it can be suggested that there is more heterogeneity in drug spending trends than in life expectancy trends across countries. The relatively large heterogeneity in pharmaceutical expenditures can be attributed to variation in health policies. However, in order to build and estimate a parametric statistical model that would allow determining if the variation in policies can explain the variation in health outcomes, a systematic collection of comparable cross-country data is needed. The data should include other factors that determine health outcomes.

Secondly, the results can be sensitive to the choice of the model. As argued by Grootendorst et al. (9), the analysis of the influence of healthcare expenditure on life expectancy is highly sensitive to model specification. He demonstrated that the introduction of some seemingly innocuous changes in the model specification leads to large changes in the parameter estimates or even obtaining estimates with different signs. It is concluded that it is difficult to estimate the parameters of the relationship between life expectancy and its determinants using aggregate data.

Thirdly, there is a number of confounding variables that can distort the causal link between healthcare spending and life expectancy (12). The most important one is GDP's growth which implies higher healthcare spending but also has an indirect impact on determinants of mortality such as better nutrition, less air pollution or better infrastructure. Life habits such as smoking or obesity are another important group of confounding variables. Finally, the causal link between healthcare spending and life expectancy can be distorted by reverse causality (increase in life expectancy may positively impact GDP and hence healthcare spending or a higher GDP may increase healthcare spending and hence improve health outcomes).

Lastly, the results presented in this paper should be treated with caution due to the limited number of analyzed health outcomes. In addition to the life expectancy, other studies (4-8) took into account infant mortality, life expectancy at birth or potential years of life lost. Consequently, no conclusions can be formulated regarding the impact of healthcare expenditures on other health measures.

CONCLUSIONS

The results of the study indicate that there are common long-term trends in life expectancies and *per capita* GDP as well as pharmaceutical and non-pharmaceutical healthcare expenditures. These common trends can be determined by a variety of factors, including life style and environmental factors which are correlated with life expectancy and income level. The dataset under investigation did not allow establishing the existence of cause-effect relationship between life expectancy and health expenditures. Significant positive short-term relations between pharmaceutical expenditures and life expectancies in OECD countries were found.

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SHORT COMMUNICATION

IN VITRO ANTHELMINTIC EFFICACY OF NATIVE PLANTS AGAINST *HAEMONCHUS CONTORTUS*

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Abstract: The current study aimed to investigate *in vitro* anthelmintic efficacy of two medicinally important plants against *Haemonchus contortus* in small ruminants. Fruit peel of *Punica granatum* Linn. (vern. Anar), leaves and roots of *Berberis lycium* Royle (vern. Sumbal) were tested for their anthelmintic efficacy. Methanolic extracts of the test plants from various plant parts were tested for anthelmintic efficacy against the *Haemonchus contortous* using albendazole as a reference standard. The results revealed that both the plant extracts exhibited potent anthelmintic activity at concentrations higher than 50 mg/mL when tested against their respective standard drug. In case of *Berberis lycium* Royle when the results were compared, methanolic roots extracts showed more potent activity as compared to leaves extracts at the same concentration. It was observed that the *in vitro* anthelmintic potential of *Punica granatum* Linn. fruit peel and *Berberis lyceium* Royale root can be used to treat helminth infections after *in vivo* trails.

Keywords: anthelmintic activity, small ruminants, *Punica granatum, Berberis lycium,* phytomedicine

Small ruminant farming has a prominent role in the sustainability of rural communities around the world (1), as well as being socially, economically and politically highly significant at national and international levels, as with all livestock species (2). The factors that negatively affect the livestock production, infections with parasites and in particular with gastrointestinal nematodes continue to represent a serious challenge to the health, welfare, productivity and reproduction of grazing ruminants throughout the world (2). Helminthiasis is a term stating to various types of parasitic worms that inhibit inside the body of humans and small ruminants and adversely effects the immune system of the host (1). Helminthiasis has an antagonistic effect on production of small ruminants and hence, causes heavy economic losses especially in developing countries including Pakistan, where mismanagement and poor control practices are prevalent (3). Controlling the helminthiasis can result in greater economic productivity caused by better ruminant growth and thus directly improve the well being of animal farmers.

Anthelmintics are used to overcome the problem of gastrointestinal parasites. Due to the poor use of conventional anthelmintics, resistance has been developed in many parasitic strains (4). Plants are used as medicines by humans since the ancient times. In the early stages of human civilization medicinal plants have been used to cure various diseases (5). To contest parasitism in many parts of the world, medicinal plants have been used for centuries and their usage is reported throughout the world till present day as in Asia (6) and Africa (7). *Haemonchus contortus* is one of the major gastrointestinal pathogens of small ruminants (8). This species was used by several authors to evaluate the anthelmintic effects of various medicinal plant species (9-12).

Natural flora is quite rich in biogenic compounds which serve as useful bioresources for the extraction of herbal medicines. Kumar et al. (13)

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extracted anthelmintic extracts from three plant species viz. *Amaranthus spinosus, Amaranthus caudatus* and *Amaranthus viridis* L. belonging to the Amaranthaceae family. Piperazine was used as a reference standard at a concentration of 10 mg/mL. *Berberis lycium* (*B. lycium*) Royle (family: Berberidaceae), a native to Pakistan, India and whole region to Himalayas is widely used like food and in folk medicine (14). *B. lycium* contains berberine, plamitine, berbamine, vitamin C, saponins, β-carotene, and various minerals including sodium and postassium (15). Previously, *B. lycium* showed antimicrobial activities (16). *Punica granatum* (*P. granatum*) belongs to taxonomic family Lythraceae, and has a number of medicinal uses. Its common name is pomegranate. Pomegranate juice, seed oil and aerial part extracts contain vitamin C, ellagic acid, quercetin, and rutin (17). Pomegranate has been used for thousands of years to cure a wide range of diseases across different cultures and civilizations. It has great nutritional values and numerous health benefits. Pomegranates are used as treatment for cancer, osteoarthritis and other diseases. The pomegranate has been used in natural and holistic medicine to treat sore throats, coughs, urinary infections, digestive disorders, skin disorders, arthritis, and to expel tapeworms.

Keeping in view the role of medicinal plants having anthelmintic efficacy; the present study was conducted to screen the anthelmintic efficacy of native plants of Sub Himalayan regions of Pakistan.

MATERIALS AND METHODS

The study area

Plant samples were collected from Sub Himalayan regions of Pakistan, situated between North Latitude 33° 65' 714" and East latitudes of 73° 03' 008" in Rawalpindi District at an elevation of 1523 m above sea level (18).

Collection of plant material and identification

The plant samples were collected in the month of October. The taxonomic position of the collected plants was identified and authenticated by the Department of Botany, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi.

Preparation of methanolic plant extracts

The collected plant samples were brought to the Applied Biotechnology and Genetic Engineering Lab, International Islamic University, Islamabad, Pakistan for further studies. The required plant material was roots and leaves in case of *B. lycium* and fruit peel in case of pomegranate. Required parts were separated by using fine cutter. They were washed thoroughly with running tap water followed by autoclaved distilled water. The samples were well dried under shade. Each part was coarsely powdered to get one kilogram chopped plant material, which was soaked in 5 L of methanol for 2 weeks in a glass container. The suspension was shaken three times a day. After 15 days, the suspension was filtered in muslin cloth and this practice was repeated three times. After getting clear filtrate, 500 mL of the filtrate was subjected to rotary evaporation maintaining the bath temperature at 40°C followed by transfer of the obtained extract (about 245 mL) to beaker (500 mL). On subjecting to further evaporation on water bath at 100°C, a gelly mass weighing about 43 g was achieved. The crude extracts of each part were labeled and stored in refrigerator for further study (18).

Collection of adult parasites

Adult parasites *(Haemonchus contortous*) from the abomasum of freshly slaughtered sheep and goat were collected from a local attributor and brought them to laboratory. These parasites were thoroughly washed with tap water followed by distilled water. The clean parasites were placed in PBS in incubator at 27°C as long as *in vitro* trails were started.

Anthelmintic efficacy

Anthelminthic activity of two medicinal plants such as methanolic extract of fruit peel of *P. granatum* Linn. and methanolic extract of roots and leaves of *Berberis lyceium* Royle was evaluated by using the assay described by Ajaiyeoba et al. (19) with certain modifications. The worms were distributed into 6 groups. The worms were distributed into 6 groups for the three categories of methanolic extract tested (fruit peel of *P. granatum* Linn., leaves and root of *Berberis lycium* Royle). First group was treated with normal saline and was used as a control. Second group was treated with anthelminthic drug suspension and albendazole was used as a reference standard. Remaining four groups were used as tests and were treated with four different concentrations of methanolic extract. All the test suspensions were prepared freshly before starting the experiment. The parameters studied were paralysis time (PT) and death time (DT). Time of paralysis was noted when the parasites were shaken vigorously and no movement of any type could be observed. Death time was recorded when motility of the parasites was completely lost and their body colors were faded away. All the results were expressed as the mean ± standard deviation (SD) of six animals in each group.

Statistical analysis

For statistical analysis ANOVA statistical significance test LSD was employed using SPSS on data to draw conclusion. Difference between means was considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Results showing the anthelmintic efficacy of methanolic extract of fruit peel of *P. granatum* Linn, are summarized in Table 1. At 15 mg/mL, mean time of paralysis was 54.67 and 69.33 min death time was recorded, whereas reference drug albendazole showed 21.67 min paralysis and 38.67 min death time at the same concentration. For 25 mg/mL, 46.67 mean time of paralysis and 61.67 min death time was observed. At the concentration of 50 mg/mL, the mean time of paralysis was reduced to 39 min and and to 57 min of death time which was comparable with the standard. The results also showed that activity was dose dependent giving the shortest mean time 32.33 min of paralysis and 42 min fot mean death time with concentration 75 mg/mL. Methanolic extracts at concentration of 75

Table 1. Anthelmintic efficacy of methanolic extract of fruit peel of *Punica granatum* Linn.

Treatment (mg/mL)	Mean time of paralysis (min) ± SD	Mean time of death (min) ± SD
Albenzadole 15	21.67^{d} ± 0.88	38.67^{c} ± 0.89
CME 15	54.67^{b} ± 1.45	69.33^{a} ± 0.89
25	46.67^{c} ± 1.42	61.67^{a} ± 0.78
50	39.00^{d} ± 0.77	57.00^{b} ± 0.89
75	32.33^{c} ± 1.15	42.00^{b} ± 1.17
Control (normal saline)	0.00 ± 0.00	0.00 ± 0.00

Note: Values having the same superscripts means statistically non-significant difference.

Table 2. Anthelmintic efficacy of crude methanolic leaves extract of *Berberis lycium* Royle

Treatment (mg/mL)	Mean time of paralysis (min) ± SD	Mean time of death (min) ± SD
Albenzadole 15	21.67^{d} ± 0.88	38.67^{b} ± 0.89
CME 15	47.33^{b} ± 0.81	65.00^{a} ± 1.19
25	36.33^{d} ± 0.57	56.33^{a} ± 0.88
50	31.00^{d} ± 0.57	51.00^{a} ± 0.57
75	23.33^{d} ± 0.20	41.67^{b} ± 0.88
Control (normal saline)	0.00 ± 0.00	0.00 ± 0.00

Note: Values having the same superscripts means statistically non-significant difference.

Table 3. Anthelmintic efficacy of crude methanolic root extract of *Berberis lycium* Royle.

Treatment (mg/mL)	Mean time of paralysis (min) ± SD	Mean time of death (min) ± SD
Albenzadole 15	21.67^{d} ± 0.88	38.67^{b} ± 0.88
CME 15	39.00^{b} ± 1.15	59.00^{a} ± 0.57
25	33.33^{d} ± 0.88	52.00^{a} ± 0.44
50	27.67^{d} ± 1.20	45.33^{a} ± 0.71
75	22.00^{c} ± 0.57	38.33^{d} ± 0.67
Control (normal saline)	0.00 ± 0.00	0.00 ± 0.00

Note: Values having the same superscripts means statistically non-significant difference.

mg/mL were most effective when compared with the reference drug against *Haemonchus contortus*. Our results are in agreement with the number of earlier studies from different regions (20-22).

Anthelmintic efficacy of crude methanolic leaves and roots extract of *B. lycium* Royle (Sumbal)

In the present study, the second plant to evaluate for anthelmintic efficacy was *B. lycium* Royle. Its leaves and roots were separately tested against the parasite. Leaves exhibit dose dependent activity. At concentration 15 mg/mL the mean paralysis time was recorded as 47.33 min whereas mean time taken by parasites for death was 65 min (Table 2). The standard drug albendazole caused paralysis at 21.67 min and death at 38.61 min for the same concentration of 15 mg/mL. At concentration 25 mg/mL, the mean time for paralysis was 36.33 min and death at 56.33 min. whereas at concentration 50 mg/mL the mean time for paralysis was recorded as 31 min and death at 51 min, which was comparable with the standard drug albendazole at the same concentration. The leaves extract showed the highest activity at 75 mg/mL with the mean paralysis time of 23.33 min and death time 41.67 min. The results suggested that leaf extract of Sumbal is more effective than the synthetic drug to kill the parasites at concentrations greater than 50 mg/mL.

Table 3 showed that the anthelmintic efficacy of root extract is dose dependent. The shortest mean time for paralysis (22 min) and death (38.33 min) was observed at concentration 75 mg/mL. The concentration at 15, 25 and 50 mg/mL showed the mean time of paralysis at 39, 33.3 and 27.67 min, respectively. The mean death rate at the same concentrations was recorded as 59, 52 and 45.33 min, respectively. Data revealed that at 25 mg/mL the activity was comparable with that of standard drug whereas it increases with an increase in concentration.

In the present study, it was observed that crude methanolic extracts of fruit peel of *P. granatum* Linn. exhibit positive response to certain degree of anthelmintic efficacy against *H. contortus*. At concentration higher than 50 mg/mL, extracts exhibited more effective activity. Our results are in agreement with the number of earlier studies from different regions (20-22) but there is no report on the native plant of Sub Himalayan regions of Pakistan. Different times of paralysis were observed for various trials as presented in Tables 1-3. This can be explained on the basis of strain differences employed in the experiment.

Our results and earlier reports revealed that the peel extract of *P. granatum* Linn. possesses potent anthelmintic efficacy irrespective of their origin and can be used as a very good replacement of synthetic drug. It is also observed that methanolic root extracts of *B. lycium* are more effective than the leaf extracts. *B. lycium* Royle contains an active alkaloid berberine. Berberine has already been reported to have promising anti-inflammatory (23), antineoplastic (24), hypoglycemic and immunomodulating (25) activities.

However, there are very few reports on the presence of anthelminthic efficacy in this plant, especially from Pakistan. Our results suggested that due to the presence of anthelminthic efficacy in this plant it can be used to develop broad spectrum drugs. Berberine has a capacity to form complexes with DNA and topoisomerase (26). It has also been reported that compounds that are cytotoxic or have the capability to interact with DNA typically show antiparasite activity. The anthelminthic efficacy observed in the methanolic extracts of this plant may be due to the ability of berberine to interact with DNA of parasites.

CONCLUSION

It was concluded that methanolic extracts of both the plants possess potent anthelmintic efficacy. However, *in vitro* anthelmintic potentials of *P. granatum* Linn. fruit peel and *B. lycium* Royle root were vivid and could be used for treating helminth infections after *in vivo* trails. The infection in small ruminants can be controlled by the cultivation of these plants in the sheep-goat's management areas.

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