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ANALYSIS

IMPACT OF STRESS FACTORS ON OPTICAL ISOMERISM OF BENAZEPRIL HYDROCHLORIDE

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Abstract: Benazepril hydrochloride contains two stereogenic centers, but is currently available as single enantiomer (S,S configuration) for the treatment of hypertension. Its enantiomer (R,R configuration) and the diastereoisomeric pair (R,S and S,R) can be regarded as impurities. Stereochemical stability of S,S isomer of benazepril hydrochloride and its potential susceptibility to conversion in the active substance and in Lisonid tablets were examinated. The separation with the use of the TLC method with the following system: chromatographic plates Chiralplate and a mobile phase: methanol – acetonitrile – 1 mM copper(II) acetate (4 : 2 : 4, v/v/v) with saturation of glacial acetic acid for 1 h and the HPLC method system: Chiral AGP column (150 × 4.0 mm × 5 μm) and a mobile phase: phosphate buffer pH = 6.0 – methanol (80 : 20, v/v) were obtained. Active substance – benazepril hydrochloride and Lisonid tablets 20 mg were subjected to the impact of different stress factors. Samples were examined after 1 and 6 weeks. It was found that none of the applied stress factors caused the transformation of the S,S enantiomer of benazepril hydrochloride in the substance and tablets to other identified stereoisomers – only the compound decomposition has occurred.

Keywords: hypertension, angiotensin converting enzyme inhibitors (ACE inhibitors), optical isomerism, HPLC, TLC, benazepril hydrochloride

Chemical compounds containing chiral centers in their molecules show optical isomerism. As a result, they may be in form of enantiomers or diastereoisomers. Enantiomers have the same dimensions and shapes as well as the same physical and chemical properties. Diastereoisomers, on the other hand, differ in physical and chemical properties. Having the present state of knowledge, it is significant to define the activity direction of enantiomers existing as medicinal products. It is possible that all identified enantiomers have identical bioactivity; it is a different situation when only one isomer is bioactive, others are inactive. It is important to define an optical purity in chiral medicines and develop analytical methods aimed at dividing enantiomers (1, 2).

Angiotensin converting enzyme inhibitors (ACE inhibitors) used in arterial hypertension treatment form a large group of medical substances containing chiral centers.

Hypertension is one of the most common diseases of the circulatory system. Due to the fact that this disorder is very widespread, it is a factor in artherosclerosis and its clinical forms: coronary disease, cardiac arrest, apoplexy, it is perceived as a social disease.

ACE inhibitors are currently a group of "first choice drugs" used in the therapy of arterial hypertension. They may be applied in treating ischemic disease and cardiac insufficiency. Their active metabolites created as a result of hydrolysis, cause the decrease in ACE activity (and related reduction of angiotensin II biosynthesis), the decrease of the release of aldosterone (as a result of lowering the concentration of angiotensin II), the increase in the concentration of vasodialysic kinin and prostaglandin as well as an indirect decrease in the synthesis of catecholamines and overall sympathetic activity. The effect of this action is the relaxation of

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vessels and diminution of the volume of circulating blood, which leads to a decrease of pressure and peripheral resistance (3, 4).

This group of medicines include such compounds as: benazepril, chinapril, cilazapril, enalapril, imidapril, fosinopril, captopril, lisinopril, moexipril, perindopril, ramipril, spirapril, trandolapril and zofenopril, in form of bases and salts of various acids.

From the recent literature on the subject, it can be concluded that several methods were used for the separation and determining enantiomers of chiral compounds, belonging to the group of ACE inhibitors in pharmaceutical products.

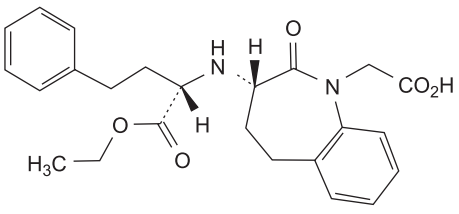
The authors applied mostly electrochemical methods along with the use of enanotioselective membrane electrodes. In this manner there determined enantiomers of: captopril (5, 6), perindopril (7, 8) as well as enalapril and ramipril (8).

The other method applied to separate isomers was the method of HPLC (9, 10). Diastereoisomers of captopril were determined in the column Chirobiotic T by Aster with application of detectors DAD or MS (9).

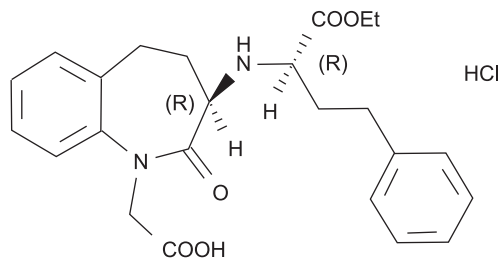
In the available literature, there was only one work on the separation of enantiomers of benazepril. The determination was performed applying a HPLC method wth the column Chiral AGP and the use of DAD detector (10).

The subject of the studies is benazepril hydrochloride, belonging to the group of ACE inhibitors. This compound contains two stereogenic centers, but is currently available as a single enantiomer (S,S configuration) for the treatment of hypertension. Its enantiomer (R,R configuration) and the diastereoisomeric pair (R,S and S,R) can be regarded as impurities. Chemical names are listed below.

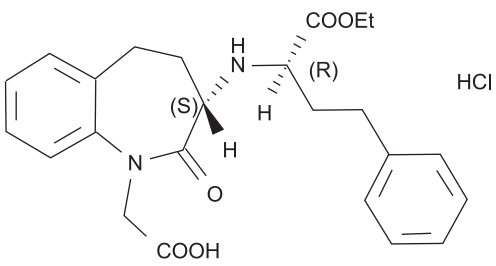
Enantiomers may differ in biological activity as well as therapeutic, pharmacological and pharmacokinetic effects. It is significant to acquire knowledge on susceptibility of chiral compounds to activity of factors which cause enantiomerization. The aim of this study was to estimate stereochemical stability of S,S isomer of benazepril hydrochloride as well as to examine its potential susceptibility to conversion in the active substance and in Lisonid tablets.



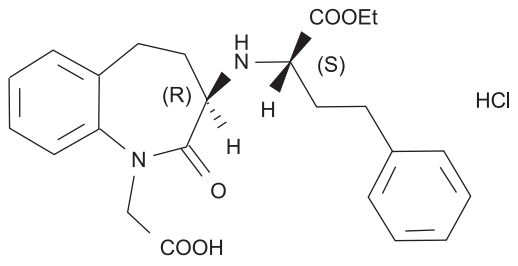
BENAZEPRIL HYDROCHLORIDE – S,S
[(3S)-3-[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl] amino]-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl] acetic acid hydrochloride



BENAZEPRIL HYDROCHLORIDE – R,R
[(3R)-3-[[(1R)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl] acetic acid hydrochloride



BENAZEPRIL HYDROCHLORIDE – S,R
[(3S)-3-[[(1R)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl] acetic acid hydrochloride



BENAZEPRIL HYDROCHLORIDE – R,S
[(3R)-3-[[(1R)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-1-yl] acetic acid hydrochloride

EXPERIMENTAL

Materials for analysis and instrumentation

Analytically pure and high purity reagents for HPLC by Rathburn. Modifiers: sodium salt of sulfobutylether β-cyclodextrin (SBE β-CNNa), tetrabutylammonium bisulfate ($TBAHSO_4$).

Liquid chromatograph Dionex *Ultimate 3000* with a spectrophotometric detector. Liquid chromatograph Shimadzu with a spectrophotometric detector. UV lamp produced by Hanau. 1000 W UV lamp. Drier produced by Memmert.

Chromatographic plates: silica gel 60 F_{254} by Merck, Silica gel RP 18 F_{254S} by Merck, Chiralplate to separate enantiomers (Silica gel RP with Cu^{2+}) by Macherey-Nagel.

HPLC columns by Daicel Chemical Industries: Chiralcel OD (250 × 4.6 mm × 10 μm), Chiralcel OC (250 × 4.6 mm × 5 μm), Chiralcel OJ (250 × 4.6 mm × 5 μm), Chiraspher NT (250 × 4.0 mm × 5 μm), Chiral AGP (150 × 4.0 mm × 5 μm).

Reference materials

Benazepril hydrochloride (S,S enantiomer) Ref. St. s. 959CEE, CGP 42454 (R,S diastereoisomer) Ref. St. s. CGP 42454-A/II, CGP 42456 (R,R enantiomer) Ref. St. s. CGP 42456-A/II, CGS 14829 (S,R diastereoisomer) Ref. St. s. CGS 14829-A/II.

Active substance

Benazepril hydrochloride s. 08/94 (Novartis).

Medicinal product

Lisonid – coated tablets 20 mg s.117885 (Pharma Arzneimittel GmbH).

Methods of separation and determination of enantiomers of benazepril hydrochloride

Thin layer chromatography (TLC)

At the first stage of the studies, optimal systems for TLC, providing separation of enantiomers of the studied compound were searched.

The following standard solutions were prepared: benazepril hydrochloride – S,S enantiomer, CGP 42454 – R,S diastereoisomer, CGP 42456 – R,R enantiomer and CGS 14829 – S,R diastereoisomer with concentration of 1 mg/mL in methanol and the mixture of all isomers with concentration of 1 mg/mL each.

On chromatographic plates: silica gel 60 F_{254}, silica gel RP 18 F_{254S} and Chiralplate (2 cm from the edge and 2 cm from the bottom) there was transferred 10 μg of each of the isomers separately and their mixture. The plates were developed in various mobile phases, both with modifiers and without them:

I. chromatographic plates silica gel 60 F_{254} and mobile phase (with modifier): acetonitrile – 0.5% SBE β-CNNa – 0.02 M $TBAHSO_4$ (8 : 1 : 1, v/v/v);
II. chromatographic plates silica gel 60 F_{254} and mobile phase (with modifier): acetonitrile – 0.5% SBE β-CNNa (8 : 2, v/v);
III. chromatographic plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa (1 : 1, v/v);
IV. chromatographic plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa – triethylamine (20 : 20 : 0.1, v/v/v);
V. chromatographic plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa – trifluoroacetic acid (20 : 20 : 0.1, v/v/v);
VI. chromatographic plates Chiralplate and mobile phase: methanol – acetonitrile – water (2 : 3 : 5, v/v/v);

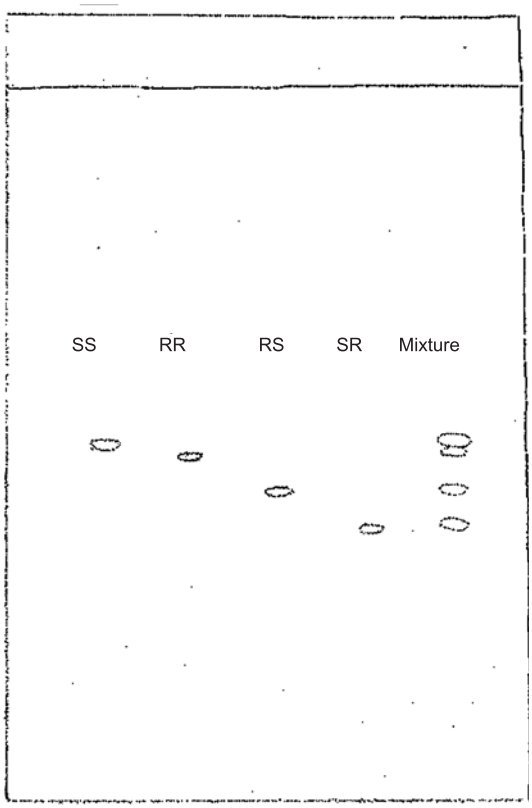


Figure 1. Chromatogram (TLC) of benazepril hydrochloride enantiomers (System IX)

Table 1. Rf values for tested enantiomers of benazepril hydrochloride in selected chromatographic systems.

Benazepril hydrochloride enantiomers	System I Rf	System II Rf	System III Rf	System IV Rf	System V Rf	System VI Rf	System VII Rf	System VIII Rf	System IX Rf
S,S enantiomer	0.80	0.80	0.26	0.29	0.30	0.39	0.57	0.42	0.44
R,R enantiomer	0.79	0.79	0.25	0.30	0.33	0.36	0.55	0.38	0.42
S,R diastereoisomer	0.84	0.84	0.25	0.26	0.33	0.34	0.50	0.33	0.36
R,S diastereoisomer	0.84	0.83	0.26	0.26	0.34	0.29	0.50	0.29	0.31

Chromatographic systems: I. plates silica gel 60 F_{254} and mobile phase (with modifier): acetonitrile – 0.5% SBE β-CNNa – 0.02 M $TBAHSO_4$ (8 : 1 : 1, v/v/v); II. plates silica gel 60 F_{254} and mobile phase (with modifier): acetonitrile – 0.5% SBE β-CNNa (8 : 2, v/v); III. plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa (1 : 1, v/v); IV. plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa – triethylamine (20 : 20 : 0.1, v/v/v); V. plates silica gel RP 18 F_{254S} and mobile phase (with a modifier): acetonitrile – 0.5% SBE β-CNNa – trifluoroacetic acid (20 : 20 : 0.1, v/v/v); VI. plates Chiralplate and mobile phase: methanol – acetonitrile – water (2 : 3 : 5, v/v/v); VII. plates Chiralplate and mobile phase: methanol – acetonitrile – water – triethylamine (20 : 30 : 50 : 0.5, v/v/v/v); VIII. plates Chiralplate and mobile phase: methanol – acetonitrile – water – trifluoroacetic acid (20 : 30 : 50 : 0.5, v/v/v/v); IX. plates Chiralplate and mobile phase: methanol – acetonitrile – water – 1 mM copper(II) acetate (4 : 2 : 4, v/v/v) with saturation with glacial acetic acid for one hour.

VII. chromatographic plates Chiralplate and mobile phase: methanol – acetonitrile – water – triethylamine (20 : 30 : 50 : 0.5, v/v/v/v);

VIII. chromatographic plates Chiralplate and mobile phase: methanol – acetonitrile – water – trifluoroacetic acid (20 : 30 : 50 : 0.5, v/v/v/v);

IX. chromatographic plates Chiralplate and mobile phase: methanol – acetonitrile – water – 1 mM copper(II) acetate (4 : 2 : 4, v/v/v) with saturation with glacial acetic acid for one hour.

After air drying of the plates, the spots positions were determined in 254 nm UV light.

TLC chromatogram of separating enantiomers of benazepril hydrochloride in the system IX is presented in Figure 1.

The obtained Rf values for the studied enantiomers are presented in Table 1.

High-performance liquid chromatography (HPLC)

At the next stage of the studies, an optimal system providing the separation of enantiomers of benazepril hydrochloride with HPLC was sought.

Standard solutions were prepared: benazepril hydrochloride (S,S enantiomer), CGP 42454 (R,R enantiomer), CGP 42454 (R,S diastereoisomer) and CGS 14829 (S,R diastereoisomer) with concentration of 1 mg/mL in methanol.

On chromatographic columns: Chiralcel OC, Chiralcel OD, Chiralcel OJ, Chiraspher NT and Chiral AGP 10 μL of each of prepared methanol solutions of enantiomers and diastereoisomers was transferred, using various mobile phases.

The separation (partial or total) of particular enantiomers was obtained in the following three HPLC systems:

I. Chiralcel OD column (250 × 4.6 mm × 10 μm), mobile phase: hexane – isopropanol – trifluoroacetic acid (850 : 150 : 1, v/v/v), column temperature: 30°C, flow rate: 1 mL/min, wavelength λ = 254 nm;

II. Chiralcel OC column (250 × 4.6 mm × 5 μm), mobile phase: hexane – isopropanol – trifluoroacetic acid (850 : 150 : 1, v/v/v), column temperature: 30°C, flow rate: 0.8 mL/min, wavelength λ = 254 nm;

III. Chiral AGP (150 × 4.0 mm × 5 μm), mobile phase: phosphate buffer pH = 6.0 – methanol (80 : 20, v/v), column temperature: 30°C, flow rate: 0.9 mL/min, wavelength λ = 240 nm.

The obtained retention times for particular enantiomers are presented in Table 2.

Total separation of studied enantiomers was obtained in system III (Fig. 2) for a mixture of all enantiomers of benazepril hydrochloride: S,S (con-

Table 2. Retention times of benazepril hydrochloride enantiomers in selected HPLC systems.

Benazepril hydrochloride enantiomers	Retention times in HPLC System I [min]	Retention times in HPLC System II [min]	Retention times in HPLC System III [min]
S,S enantiomer	21.0	9.7	6.9
R,R enantiomer	15.5	7.8	13.0
S,R diastereoisomer	12.5	10.5	15.3
R,S diastereoisomer	15.0	9.6	25.4

Systems: I. Chiralcel OD column (250 × 4.6 mm × 10 µm), mobile phase: hexane – isopropanol – trifluoroacetic acid (850 : 150 : 1, v/v/v), column temp. 30°C, flow rate 1 mL/min, λ = 254 nm; II. Chiralcel OC column (250 × 4.6 mm × 5 µm), mobile phase: hexane – isopropanol – trifluoroacetic acid (850 : 150 : 1, v/v/v), column temp. 30°C, flow rate 0.8 mL/min, λ = 254 nm; III. Chiral AGP (150 × 4.0 mm × 5 µm), mobile phase: phosphate buffer pH = 6.0 – methanol (80 : 20, v/v), column temp. 30°C, flow rate: 0.9 mL/min, λ = 240 nm.

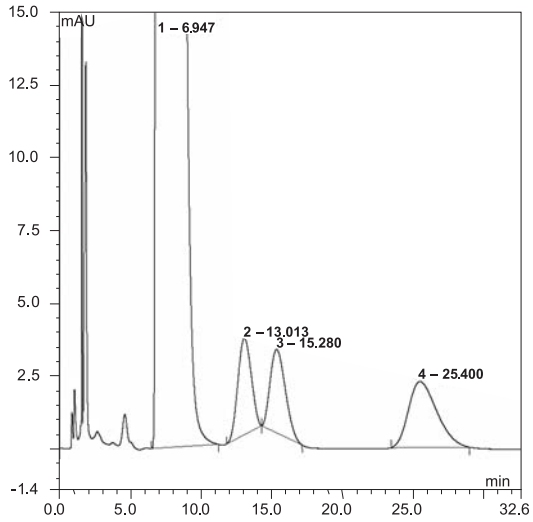


Figure 2. Chromatogram (HPLC) of benazepril hydrochloride enantiomers (System III)

centration of 1 mg/mL) and R,R, R,S and S,R (concentration of 0.01 mg/mL) on the column Chiral AGP.

In the systems I and II only partial separation of enantiomers was obtained.

Susceptibility of benazepril hydrochloride to stereoconversion for active substances and in tablets

The examination included the active substance of benazepril hydrochloride and coated tablets Lisonid 20 mg.

Preparation of samples

Forty milligrams of benazepril hydrochloride and amount of mass tablet of Lisonid, adequate to 40 mg of active substance, (mass of ca. 2 tablets) were subjected to the impact of different stress factors:

1. 1 M sodium hydroxide solution (10 mL) and temperature 40°C;
2. 0.05 M sodium hydroxide solution (10 mL) and temperature 40°C;
3. 1 M hydrochloric acid solution (10 mL) and temperature 40°C;
4. 6% hydrogen peroxide solution (10 mL) and temperature 40°C;
5. temperature 40°C;
6. 1000 W UV light, 3 times for 10 min.

Susceptibility of benazepril hydrochloride to stereoisomerism in conditions no. 3, 4 and 5 was defined after 1 and 6 weeks; in conditions no. 1 after 1 week and in conditions no 2 after 6 weeks. Samples in conditions no. 6 were examined directly after having been exposed to UV light.

Preparation of solutions of samples and reference materials

Prepration of the mixture of enantiomers of benazepril hydrochloride

Solutions of enantiomers of benazepril hydrochloride: R,R, R,S and S,R with concentration of 0.5 mg/mL in methanol were prepared.

To a volumetric flask, 50 mg of S,S enantiomer of benazepril hydrochloride was weighed, filled with 1 mL of methanol solutions of enantiomers: R,R, R,S and S,R, 10 mL methanol were added and then filled with the mobile phase to 50.0 mL.

Final concentration of S,S benazepril hydrochloride enantiomer was 1 mg/mL, those of enantiomers R,R, R,S and S,R were 0.01 mg/mL.

Preparation of examined samples

The samples after conditions no. 1–3 were filtered through membrane filters (0.45 µm) and were

adjusted to pH ca. 5.0. Samples (2.5 mL) of solutions were transferred to volumetric flasks and filled with mobile phase to 10 mL. Initial concentration of S,S benazepril hydrochloride enantiomer was 1 mg/mL.

The samples after conditions no. 4 were filtered with membrane filters (0.45 μm). Samples (2.5 mL) of solutions were transferred to volumetric flasks and filled with a mobile phase to 10 mL. Initial concentration of S,S benazepril hydrochloride enantiomer was 1 mg/mL.

From the samples after conditions no. 5 and 6, 10 mg of substance or the amount of mass tablet equivalent to 10 mg of active substance was weighed to volumetric flasks and 1 mL of methanol was added. Samples were shaken for 5 mintes on ultrasonic bath and filled with a mobile phase to 10 mL. The samples were filtered with membrane filters (0.45 μm). Initial concentration of S,S benazepril hydrochloride enantiomer was 1 mg/mL.

For examination of the prepared solutions the conditions of system III were applied: column: Chiral AGP (150 × 4.0 mm × 5 μm); mobile phase: phosphate buffer pH = 6.0 – methanol (80 : 20, v/v); wavelength: λ = 240 nm; column temperature: 30°C; flow rate: 0.9 mL/min; injection volume: 20 μL.

DISCUSSION OF RESULTS

Knowledge on the configurational stability of substances is of utmost importance in case of chiral medicines. It is significant to estimate the stereochemical durability of enantiomers and to examine their potential susceptibility to conversion under various factors.

As a result, there was made an attempt to examine the stereochemical durability of S,S benazepril hydrochloride enantiomer in an active substance and medicinal product – coated tablets Lisonid 20 mg.

At the first stage of the study, the chromatographic methods of separation of examined enantiomers were explored applying TLC and HPLC methods.

The best separation with the use of the TLC method, was obtained with the following system: chromatographic plates Chiralplate and mobile phase: methanol – acetronitrile – 1 mM copper(II) acetate (4 : 2 : 4, v/v/v) with saturation of glacial acetic acid for 1 h.

The Rf values amounted to: S,S enantiomer – 0.44, R,R enantiomer – 0.42, S,R diastereoisomer – 0.36, R,S diastereoisomer – 0.31, respectively. (Table 1, Fig. 1).

For separation with HPLC method, several chiral columns as well as mobile phases were tested. The system, described in Ph. Eur., proved to be the best one: Chiral AGP (150 × 4.0 mm × 5 μm) and mobile phase: phosphate buffer pH = 6.0 – methanol (80 : 20, v/v), column temperature: 30°C, flow rate: 0.9 mL/min, wavelength λ = 240 nm. The obtained retention times were: S,S enantiomer – 6.9 min, R,R enantiomer – 13.0 min, S,R diastereoisomer – 15.3 min, R,S diastereoisomer – 25.4 min (Table 2, Fig. 2).

The TLC system no. IX gave only identification of enantiomers and could be used in hight-speed test of stereochemical stability research of S,S enantiomer of benazepril hydrochloride.

Method that gave the highest resolution of benazepril hydrochloride enantiomers was HPLC with Chiral AGP column. This is a validated, precise, sensitive and accurate method, that seems to be the best for identification and determination for all studied compounds.

This system was subsequently used for examination of stereochemical stability of benazepril hydrochloride in the substance and Lisonid – coated tablets 20 mg.

Active substance – benazepril hydrochloride and coated tablets Lisonid 20 mg were subjected to the impact of different stress factors named above.

In 1 M sodium hydroxide solution, at 40°C, there was a total decomposition of benazepril hydrochloride in the substance and tablets to unknown contaminants within one week. There was no transfer of S,S enantiomer into other stereoisomers.

In 0.05 M sodium hydroxide solution, at 40°C, there was a decomposition of benazepril hydrochloride to unknown contaminants, in the substance in 100% and in tablets in 95% within one week.

Benazepril hydrochloride in 1 M hydrochloric acid solution and temperature of 40°C, both in the substance and tablets underwent decomposition to unknown contaminants in 20% after one week and in 90% after 6 weeks.

The influence of 6% hydrogen peroxide solution and temperature of 40°C on the compound, resulted in the decomposition to unknown contaminants in 26% of the substance and in 95% in tablets after 1 week. After 6 weeks there was a decomposition of 50% in the substance. In tablets it remained at the same level (95%).

Benazepril hydrochloride in the substance and medicinal product at the temperature of 40°C after 1 week and after 6 weeks was stable and did not change.

Treatment with 1000 W UV light (3 × 10 min) on benazepril hydrochloride did not cause any

changes in the substance, while in tablets, there was a decomposition of 98% to unknown contaminants.

It was found that none of the applied stress factors caused the transformation of the S,S enantiomer of benazepril hydrochloride in the substance and tablets to other identified stereoisomers – only the compound decomposition occurred.

CONCLUSIONS

1. Conditions of separation of benazepril hydrochloride enantiomers were developed using TLC and HPLC.
2. The HPLC method could be used for identification and separation of isomers in the active substance and medicinal product.
3. Stability examination of benazepril hydrochloride in substance and Lisonid tablets as well as separation of stereoisomers by means of the HPLC method were performed. In the applied conditions, S,S benazepril hydrochloride enantiomer did not transform to other identified stereoisomers – only the compound decomposition occurred.

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DETERMINATION OF FLUCONAZOLE IN HUMAN PLASMA BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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Abstract: A rapid, simple and reproducible high performance liquid chromatographic method was developed and validated for determination of fluconazole in human plasma. The separation was performed on MZ C8 column (125 × 4 mm, 5 µm) using acetonitrile – potassium dihydrogen phosphate buffer (15 : 85, v/v), pH 3.0, as the mobile phase at a flow rate of 1.5 mL/min. The wavelength was set at 261 nm. The assay enables the measurement of fluconazole for therapeutic drug monitoring with a minimum quantification limit of 20 ng/mL. The method involves simple, protein precipitation procedure and analytical recovery was complete. The calibration curve was linear over the concentration range 0.1-4 µg/mL. The coefficients of variation for inter-day and intra-day assay were found to be less than 10%.

Keywords: fluconazole, HPLC, UV-detector, pharmacokinetic

Fluconazole [2-(2,4-difluorophenyl)-1,3-bis (1H-1,2,4-triazol-1-yl)-2-propanol] is a broad-spectrum triazole antifungal agent that has emerged as a suitable alternative to amphotericin B in the treatment and prevention of different superficial and systemic fungal infections (1, 2). It inhibits cytochrome P450-dependent enzymes, resulting in impairment of ergosterol synthesis in fungal cell membranes. Fluconazole is well absorbed following oral administration, bioavailability from oral route is approximately 90%. About 80% of a dose is excreted unchanged in the urine and about 11 % as metabolites (3).

Fluconazole is widely used in clinical practice. Its favorable pharmacokinetics facilitates the management of its dosing (4, 5). However, in some situations its pharmacokinetics is difficult to predict, and determination of circulating fluconazole levels is significant to guide its dosing (6–13). The treatment of these infections requires adjustments in fluconazole dosages. Finally, pharmacokinetic studies are significant in experimental treatment models (14). Since changes in the pharmacokinetics of fluconazole are still unknown, it is imperative to consider burn patients' drug levels with a controlled clinical protocol (15).

Several analytical methods including bioassay (16), gas chromatography (17), liquid chromatography (LC) with UV detection (18–22) and LC with tandem mass spectrometry (LC-MS/MS) (23, 24) have been described for quantification of fluconazole in human plasma and serum samples. Most of the presented methods need long chromatographic elution time for analysis of fluconazole in serum and sample preparation is complex and time consuming. The LC-MS/MS method is very sensitive but it is not available for most laboratories because of their apparatus requirement and financial reasons. High-performance liquid chromatography (HPLC) is preferred due to its selectivity and the specificity of the assay. In recent years, there have been wide studies for the different methods of the determination of fluconazole in biological samples by HPLC-UV (25–32). Reversed phase high-performance liquid chromatography (RP-HPLC) is an efficient method for the analysis of drugs.

This study describes a rapid and validated HPLC method using UV detection, which enables

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the determination of fluconazole with good accuracy at low drug concentrations in plasma using single-step extraction procedure. We also demonstrate the applicability of this method for pharmacokinetic studies in humans.

MATERIALS AND METHODS

Chemicals

Fluconazole and methocarbamol (internal standard) were supplied by Pars-Daru Pharmaceuticals (Tehran, Iran). Fluconazole is available as oral capsules containing 150 mg of fluconazole and other inactive ingredients. HPLC-grade acetonitrile and all other chemicals were obtained from Merck (Darmstadt, Germany). Water was obtained by double distillation process and purified additionally with a Milli-Q system (Millipore, Bedford, MA, USA).

Instruments and chromatographic conditions

The chromatographic apparatus consists of a model Wellchrom K-1001 pump, a model Rheodyne 7125 injector and a model K 2501 UV detector connected to a model Eurochrom 2000 integrator, all from Knauer (Berlin, Germany). The separation was performed on MZ C8 (125 × 4.0 mm, 5 µm) column from Merck (Darmstadt, Germany). The wavelength was set at 261 nm. The mobile phase consisted of acetonitrile – potassium dihydrogen phosphate buffer (15 : 85, v/v), pH 3.0, at a flow rate of 1.5 mL/min. The mobile phase was prepared daily and degassed by ultrasonication before use.

Standard solutions

Stock solution (5 mg/mL) of fluconazole and methocarbamol (10 mg/mL) were prepared in methanol. The working standard solutions were obtained by dilution with methanol.

Sample preparation

To 450 mL of plasma in a glass-stoppered 15 mL centrifuge tube were added 50 mL of methocarbamol as internal standard (10 mg/mL), 500 mL of acetonitrile and 100 mg NaCl. After mixing (30 s), the mixture was centrifuged for 15 min at 8000 rpm. The organic phase was separated and dried under argon. Then, 50 mL of mobile phase was added and 40 mL of supernatant was injected into liquid chromatograph.

Biological samples

Twelve male healthy volunteers were included in this study. The study protocol was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences and written informed consent was obtained from the volunteers. Fluconazole was administered in a single dose of 150 mg to the volunteers after overnight fasting. Plasma samples were collected at several intervals after dosing and then frozen immediately at –20°C until assayed.

Stability

The stability of fluconazole was assessed for spiked plasma samples stored at –20°C for up to two months and at ambient temperature for at least 24 h. The stability of stock solutions stored at –20°C was determined for up to one month by injecting appropriate dilutions of stock solution in distilled water on day 1, 15, and 30 and comparing their peak areas with fresh stock solution prepared on the day of analysis. Samples were considered to be stable, if the assay values were within the acceptable limits of accuracy and precision.

Plasma calibration curves and quantitation

Blank plasma was prepared from heparinized whole blood samples collected from healthy volunteers and stored at –20°C. After thawing, 50 µL of fluconazole working standards were added to yield final concentrations of 0.1, 0.25, 0.5, 1, 2, 3, and 4 µg/mL. Internal standard solution was added to each of these samples and the samples were then prepared for analysis as described above. Calibration curve was constructed by plotting peak area ratio (y) of fluconazole to the internal standard *versus* fluconazole concentrations (x). A linear regression was used for quantitation.

Precision

The precision of an analytical method describes the closeness of individual measures of fluconazole when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of the biological matrix. The precision is reflected by the standard deviation, while relative precision is calculated as the coefficient of variation (CV), i.e., relative standard deviation (% RSD). Calculations of precision are largely independent of the number of replicates, except that more replicates may give a better estimate of the population variance. The measure RSD can be explaining by: repeatability (intra-day precision) and reproducibility (between laboratories precision). Intra-day precision was determined by repeatability of the method with obtained data on the same day under similar conditions from the precision experiments for five multiple injections at three different

dilutions (0.8, 1.6, and 3.2 µg/mL). The concentration of the sample is then obtained by measuring the peak area and the coefficients of variation were calculated, being within the acceptable criteria of less than 0.1 (Table 1).

Inter-day precision was obtained from reproducibility of the method with obtained data by repeating the assay five times (n = 5) on five different days, by injecting standard fluconazole at three different dilutions (0.8, 1.6, and 3.2 µg/mL). The coefficients of variation value were found to be less than 0.1 (Table 2).

Accuracy

Accuracy is the degree of agreement of a measured value with an accepted reference value. The accuracy of the method is determined by replicate analysis of samples containing known amounts of the fluconazole at three different concentrations equivalent to (0.8, 1.6, and 3.2 µg/mL) of the active pharmaceutical ingredient, by adding a known amount of fluconazole standard, and calculating the recovery of fluconazole with recovery and coefficients of variation for each concentration (Table 3).

Limit of quantification (LOQ) and recovery

The limit of detection (LOD) expresses the lowest concentration of analyte that can be detected for a given type of sample, instrument, and method, but not necessarily quantified under the stated experimental conditions. The limit of quantitation (LOQ) is the lowest concentration of analyte in a sample that can be determined with acceptable accuracy (70 – 120%) and precision (< 20%). LOD and LOQ were estimated using the following formulas:

$$LOD = 3.3\sigma/S \text{ and } LOQ = 10\sigma/S$$

where σ represents the standard deviation of the response (estimated based on the calibration curve), and S is the slope of the calibration curve. The LOD and LOQ values were 20 ng/mL and 61 ng/mL,

Table 1. Results of precision study (intra-day) for the fluconazole analysis in plasma (n = 5).

Sample	Concentration (µg/mL)	Injection no.					Mean	SD	CV
		1	2	3	4	5			
Fluconazole	0.8	0.60	0.69	0.66	0.63	0.67	0.65	0.03	0.05
	1.6	1.40	1.44	1.41	1.37	1.19	1.36	0.10	0.07
	3.2	3.22	2.44	2.9043	2.83	2.77	2.83	0.28	0.10

Table 2. Results of precision study (inter-day) for the fluconazole analysis in plasma (n = 5).

Sample	Concentration (µg/mL)	Injection no.					Mean	SD	CV
		1	2	3	4	5			
Fluconazole	0.8	0.60	0.65	0.63	0.61	0.63	0.63	0.02	0.03
	1.6	1.40	1.20	1.42	1.17	1.37	1.31	0.11	0.09
	3.2	3.22	2.58	2.81	2.51	2.83	2.79	0.28	0.10

Table 3. Results of recovery study for the fluconazole analysis in plasma (n = 3).

Sample	Concentration (µg/mL)	Injection no.			Mean	STD
		1	2	3		
Fluconazole	0.8	106.75	107.92	108.36	107.68	0.83
	1.6	110.06	109.46	111.62	110.38	1.11
	3.2	112.21	109.73	110.56	110.83	1.26

respectively. The results of LOD and LOQ supported the sensitivity of the developed method.

Pharmacokinetic analysis

Fluconazole pharmacokinetic parameters were determined by non compartmental methods. Elimination rate constant (k) were estimated by the least-square regression of plasma concentration-time data points lying in the terminal log-linear region of the curves. Peak plasma concentration (C_{max}) and time to peak concentration (T_{max}) were derived from the individual subject concentration-time curves. The area under the plasma concentration-time curve from time zero to the last measurable concentration at time t (AUC_{0-t}) was calculated using the trapezoidal rule. The area was extrapolated to infinity ($AUC_{0-\infty}$) by addition of C_t/K to AUC_{0-t} where C_t is the last detectable drug concentration.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, fluconazole and the internal standard peaks were well resolved. Endogenous plasma components did not give any interfering peaks. Separation was performed on a short reversed-phase C8 column, which allows easy optimizing chromatographic conditions to obtain desirable resolution in a short time. Accordingly, the chromatographic elution step is undertaken in a short time (less than 10 min) with high resolution. Figure 1 shows typical chromatograms of blank plasma in comparison to spiked samples analyzed for a pharmacokinetic study. The average retention times of fluconazole and methocarbamol were 6.1 and 7.8 min, respectively. The peaks were of good shape and completely resolved at therapeutic concentrations of fluconazole. In addition, this separation was obtained using low amount of organic solvent in composition of mobile phase compared to related published methods. In our method, sample preparation involves protein precipitation using acetonitrile. Protein precipitation became more efficient with increasing volumes of acetonitrile. However, greater volumes of acetonitrile diluted the sample, thereby affecting the sensitivity of the assay. To improve the sensitivity, a 1:1 ratio of acetonitrile to plasma was considered for sample preparation. Under this condition, the majority of protein was precipitated and fluconazole and internal standard were free of inference from endogenous components in plasma. The calibration curve for the determination of fluconazole in plasma was linear over the range 0.1 – 4 µg/mL. The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. The correlation coefficients (r) for calibration curves were equal to or better than 0.998. The slopes of plasma standard curves in the nine different preparations were practically the same (the coefficients of variation were less than 2% for the slopes of plasma standard curves). For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves. The mean linear regression equation of calibration curve for the analyte was:

$$y = 0.778x - 0.0047$$

where y was the peak area ratio of the analyte to the internal standard and x was the concentration of the analyte. The analytical recovery from plasma at three different concentrations of fluconazole was determined. Known amounts of fluconazole were added to drug-free plasma in concentrations ranging from 0.8 to 3.2 µg/mL. The internal standard was added and the absolute recovery of fluconazole was calculated by comparing the peak areas for extracted fluconazole from spiked plasma and a standard solution of fluconazole in methanol containing internal standard with the same initial concentration. The average recovery was 109.63 ± 1.7% (n = 5) and the dependence on concentration was negligible. The recovery of internal standard, methocarbamol was almost complete at the concentration used in the assay. Using UV detection method, the limit of quantification, as previously defined, was 20 ng/mL for fluconazole. This is sensitive enough for drug monitoring and other purposes such as pharmacokinetic studies. We assessed the precision of the method by repeated analysis of plasma specimens containing known concentrations of fluconazole. As shown in Tables 1 and 2, coefficients of variation were less than 10%, which is acceptable for the routine measurement of fluconazole. Stability was determined for spiked plasma samples under the conditions as previously described. The results showed that the samples were stable during the mentioned conditions.

The aim of our study was to develop a rapid and sensitive method for the routine analysis of biological samples in pharmacokinetic fluconazole research. This method is well suited for routine application in the clinical laboratory because of the speed of analysis and simple extraction procedure. Over 350 plasma samples were analyzed by this method without any significant loss of resolution. No change in the column efficiency and back pressure was also observed over the entire study time,

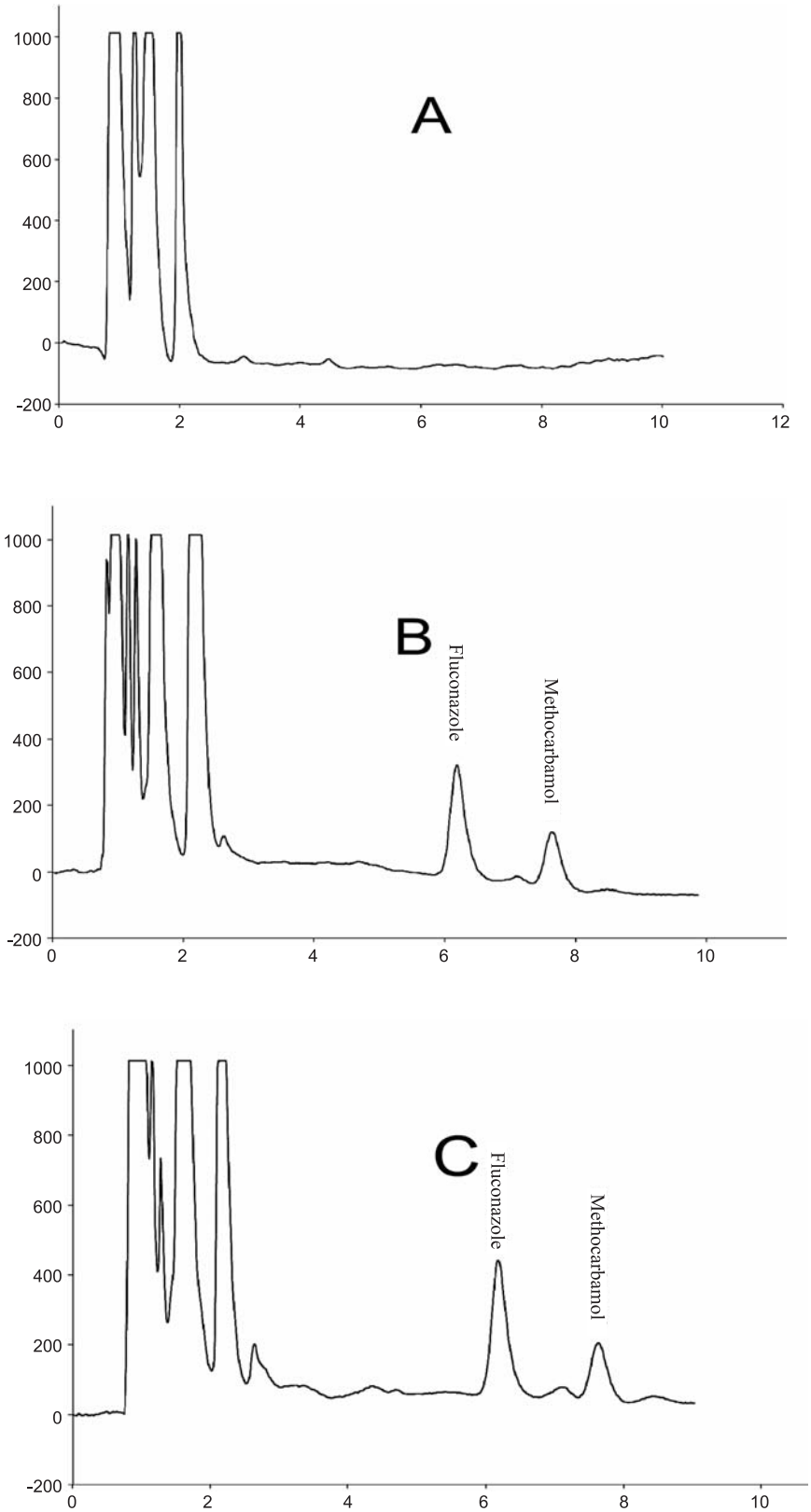

Figure 1. Chromatograms of (A) blank plasma; (B) blank plasma spiked with 1500 ng/mL fluconazole and 500 ng/mL methocarbamol (internal standard); (C) plasma sample from a healthy volunteer three hours after oral administration of 150 mg of fluconazole

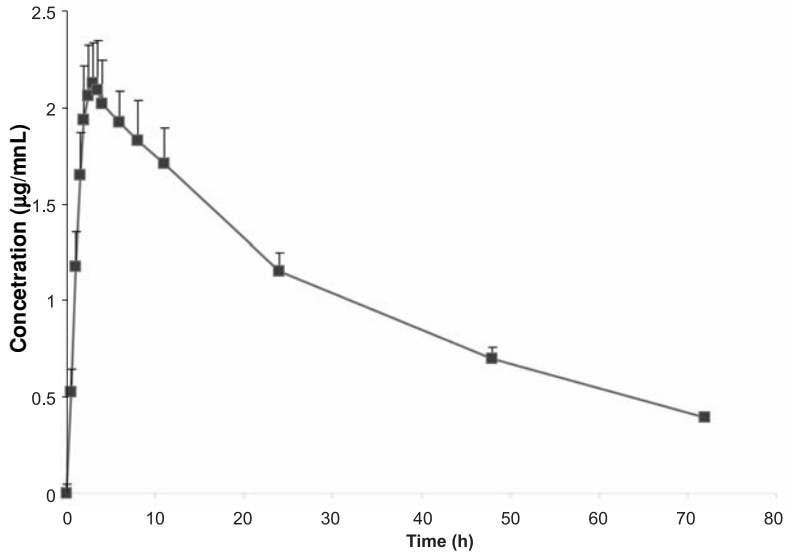

Figure. 2. Mean plasma concentration-time profile of fluconazole in healthy volunteers (n = 12) after a single 150 mg of fluconazole

Table 4. Pharmacokinetic parameters of fluconazole in healthy volunteers following a single oral dose of 150 mg of fluconazole.

Parameter	Result (mean ± SD)
T_{max} (h)	2.62 ± 0.40
C_{max} (µg/mL)	2.22 ± 0.46
AUC_{0-t} (µg×h/mL)	73.26 ± 20.29
$AUC_{0-\infty}$ (µg×h/mL)	91.18 ± 28.54
K_{el} (h^{-1})	0.024 ± 0.004

thus proving suitability of the method. In this study, plasma concentrations were determined in twelve healthy volunteers, who received 150 mg of fluconazole each. Figure 2 shows the mean plasma concentration-time profile of fluconazole. The derived pharmacokinetic parameters of 12 healthy volunteers are summarized in Table 4. These pharmacokinetic parameters are in good agreement with those found previously (4).

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LIPOPHILICITY ASSESSMENT OF SPIRONOLACTONE BY MEANS OF REVERSED PHASE LIQUID CHROMATOGRAPHY AND BY NEWLY DEVELOPED CALCULATION PROCEDURES

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Abstract: The parameters of lipophilicity of spironolactone (a single member of steroids group), which is widely applied as diuretic and antihypertensive agent, were experimentally determined by reversed-phase TLC and HPLC methods as well as calculated using different computer programs and also by a novel mode based on topological indices. Various stationary phases, such as RP-18WF_{254}, RP-2F_{254}, RP-18F_{254} and also different binary solvent systems composed of organic modifier (e.g., methanol, dioxane, acetone) and water were used as mobile phases in order to predict the following chromatographic parameters: R_{MW} and $logk_w$, respectively. LogP of examined spironolactone calculated with respective theoretical procedures: AlogPs, $logP_{KOWWIN}$, xlogP2, xlogP3, AClogP, AlogP, MlogP and also $logP_{average}$ were obtained from online package software. The partition coefficients expressed as $logP_1$, $logP_2$ and $logP_3$ were calculated by means of the formulae based on the numerical values of the following topological indices: , ^0B, ^1B, W, $^0\chi^v$ and I_B, which was novelty of this study. A good agreement between logP calculated by new method and experimentally estimated lipophilicity parameters (by chromatography and by shake flask method) was found. The results confirmed applicability of the topological indices for calculating lipophilicity of spironolactone as alternative procedure to the experimental and other computed logP values.

Keywords: lipophilicity, logP, $logk_w$, R_{MW}, spironolactone, topological indices, RP-TLC, RP-HPLC

For many years, increasing development of new biologically active compounds for application in medicine as potential drugs is observed. The pharmacokinetic profile of newly discovered drugs depends on various factors. Among different physicochemical properties that has significant impact on drug behavior in biological system is lipophilicity (hydrophobicity). This property plays decisive role in drug design, especially in the prediction of transport of biomolecule trough cell membranes in biological system. The most common lipophilicty measure is logP (logarithm of partition coefficient) determined by different separation methods including chromatography. The traditional method which has been widely used for the determination of lipophilicty (logP) of organic compounds in *n*-octanol - water system is the shake flask technique (1). As it is well known, this method is rather time consuming and allows to determine logP in limited range from -3.0 to +3.0, therefore in order to eliminate this limitation, the chromatographic methods can be utilized. Among numerous chromatographic approaches like reversed phase thin layer chromatography (RP-TLC) or reversed phase high performance liquid chromatography (RP-HPLC), which can be currently performed in lipophilicity investigations with the use of modern mobile and stationary phases, such as immobilized articificial membranes (IAM), an alternative technique to those may be micellar liquid chromatography (MLC) in TLC and HPLC systems. Electrophoretic methods are suitable for the estimation of the lipophilicity of various biomolecules in the wide range of logP.

The most commonly used chromatographic lipophilicity descriptors are: R_{MW} – in thin layer chromatography and also $logk_w$ in column chromatography. Analogously to both, the micellar $logk_m$ parameter can be evaluated as lipophilicity descriptor. Many researches were applying RP-TLC, RP-HPLC and also MLC in lipophilicity study of novel drugs with very different structures and functionalities like, for example: some oxicams from a group of nons-

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teroidal anti-inflammatory drugs, anitiproliferative 8,10-substituted quinobenzothiazines, selected phenylthioamides and 1,2,4-triazoles with antifungal activity, biologically active imidazolinum based ionic liquids, some β-blockers drugs, and also γ-butyrolactone derivatives with anticonvulsant and analgesic activity (2-15). The predicted lipophilicity parameters were found to be significantly correlated with the activities of these compounds.

Despite of the widely applied experimental techniques, such as described chromatographic methods, lipophilicity of biologically active compounds could be determined by the use of computational methods (1, 16, 17). Computed methods of prediction of logP from compound structure are still in development and show different power of calculation of this descriptor (16). From this fact arises conclusion that in order to obtain reliable lipophilicity parameter, the computed logP should be compared with those which have been obtained experimentally.

In the past decade, the electrotopological state indices and also topological indices are becoming increasingly popular for modeling of lipophilicity of different organic compounds (18-21). The current literature review demonstrates the topological approach to estimating lipophilicity of 223 heterogeneous organic compounds (21). Moreover, it was found that topological distance indices are useful descriptors for correlating a variety of biological properties (e.g., pharmacological activity) of chemical compounds in QSAR studies (22-25). For example, using the well known in literature distance-based topological indices, a QSAR analysis on the antibacterial activity of some sulfa drugs was carried out (23). In another work, similar QSAR studies on a series of imidazole derivatives as novel ORL1 receptor antagonist with the use of number of structural descriptors including topological index (Balaban Index) was performed (25).

The main goal of this experiment was to apply reversed phase TLC and HPLC to indirectly determine lipophilicity descriptor (R_{MW} and $logk_w$) of spironolactone, a single member of steroids, which has been widely applied in medicine as antidiuretic agent. The second stage was determination of other lipophilicity parameters by computational methods: AlogPs, $logP_{KOWWIN}$, xlogP2, xlogP3, AClogP, AlogP, MlogP, $logP_{average}$ and also by the newly developed procedures based on topological indices: $logP_1$, $logP_2$ and $logP_3$. The third stage was comparison and assessment of all obtained results.

The present work is a part of our extensive study on the use of the two experimental methods like TLC and HPLC and also selected theoretical procedures based on compound structure to estimate the lipophilic properties of pharmaceutically important steroids with different biological activity (26-34). In our previous investigations, various steroid compounds belonging to conjugated and unconjugated bile acids and also some steroid anabolics were investigated for lipophilic properties using RP-TLC, RP-HPTLC and also some theoretical methods. In the present research, the applicability of both techniques, including the newly developed calculating procedure based on topological indices as alternative to reference shake flask method, in studying lipophilicity of spironolactone was estimated.

EXPERIMENTAL

Reagents and materials

For the preparation of mobile phases, methanol, acetone and dioxane of HPLC grade POCh (Gliwice, Poland) and distilled water for HPLC (E. Merck, Darmstadt, Germany) in RP-TLC, RP-HPTLC and also RP-HPLC analyses were used. The standard of spironolactone (97%, No. Catalog. S3378-1G) was procured from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of standard solution

Standard solution of tested compound for RP-TLC and RP-HPTLC analysis was prepared by dissolving 10 mg of accurately weighted amount of this substance in 10 mL of methanol. Thus, final concentration of analyte was 1 mg/mL. For the purpose of RP-HPLC analysis, methanol solution of spironolactone at concentration of 3 mmol/L was utilized.

Chromatographic investigations

RP-TLC and RP-HPTLC analysis

Lipophilicity of spironolactone was evaluated by thin-layer chromatography on various stationary phases, such as 6 cm × 10 cm aluminum RP-TLC plates (RP-18F_{254}, Art. 1.05559), glass RP-HPTLC plates: RP-18WF_{254} (Art. 1.13124) and also RP-2F_{254} (Art 1.13726) manufactured by E. Merck (Darmstadt, Germany). Three microliters of examined solution was spotted onto the chromatographic plates (1 cm distance from the bottom) in each case. The chromatograms were developed with the use of mobile phases consisting of organic modifier (e.g., methanol, acetone or dioxane) - water in different volume compositions. The content of methanol, acetone and also dioxane in applied mobile phases were gradually varied by 5% (v/v) in the range from 50 to 90% (v/v).

Fifty milliliters of mobile phase was used in all cases. The chromatograms were developed at 18 ± 2°C in a 10 cm × 20 cm chromatographic chamber (Camag, Switzerland) which has been previously saturated with solvent vapors during 30 min. The development distance was 8 cm. After developing, the chromatographic plates were dried at 18 ± 2°C using a fume cupboard. Spectrodensitometric scanning was done using a Camag TLC Scanner 3 (Muttenz, Switzerland) which was controlled by WinCATS 1.4.2 software. All spectrodensitometric measurements were conducted in reflectance absorbance mode at wavelength of 238 nm. This wavelength was an optimum for examined spironolactone, and hence, it was selected for densitometric analysis. The source of radiation was a deuterium lamp. The scanning speed was 20 nm/s and the data resolution was 100 µm/step. The slit dimension was kept at 10.0 mm × 0.40 mm, Macro. Each analysis was repeated three times. Mean R_F value was used to calculate R_M.

Reversed-phase high performance liquid chromatography (RP-HPLC)

The compound was examined using a chromatograph HPLC Hewlett Packard 1050 (Canada) with the UV detector. The chromatographic conditions of applied HPLC method were as follows: the column C-18 (Eurospher 100-5) of the size 250 × 4 mm, packing of a 5 µm diameter, additionally equipped with precolumn (Knauer, Germany). The injection volume was 10 µL, the eluent flow was 1 mL/min. The detection of spironolactone was conducted at 238 nm. The isocratic elution of separated compound was carried out by the use of mobile phases: methanol – water and also dioxane – water. The content of methanol and dioxane in mobile phase was gradually varied by 5% (v/v) in the range from 55-95% (v/v). The t_R values are mean value from three independent analyses.

Lipophilicity parameters

Chromatographic parameter of lipophilicity (R_{MW})

For subsequent calculations, mean R_F values obtained under applied chromatographic conditions (various mobile phases and stationary phases) were converted to retention parameter R_M according to the expression:

$$R_m = \log(\frac{1}{R_F} - 1) \quad [1]$$

Linear correlation between R_M and volume fraction of organic modifier in mobile phase (φ) permits the extrapolation of obtained R_M values to the zero concentration of organic modifier (methanol, acetone or dioxane) in accordance with Soczewiński-Wachtmeister equation [2] and estimate relative retention parameter R_{MW} (1):

$$R_M = R_{MW} - S \times \varphi \quad [2]$$

where: R_M is the R_M value of spironolactone, R_{MW} is the R_M value extrapolated to zero concentration of organic modifier in mobile phase, S is the slope of the regression plot, φ is the volume fraction of organic modifier in mobile phase used (e.g., methanol, acetone, dioxane).

Chromatographic parameter of lipophilicity ($logk_w$)

The logarithm of retention factor logk of examined spironolactone obtained under applied solvent systems was calculated from retention time (t_R) determined by means of RP-HPLC method according to the formula:

$$\log k = \log \frac{t_R - t_M}{t_M} \quad [3]$$

where: t_R and t_M – is the retention time [min] of spironolactone and also dead-time, respectively.

For each mobile phase, the logk walue was determined and then the extrapolation of obtained logk to zero content of organic modifier (methanol and dioxane) in mobile phase: methanol - water and dioxane -to water accordance with Snyder-Soczewiński equation allowed obtain the $logk_w$ (1):

$$\log k = \log k_w - S \times \varphi \quad [4]$$

Table 1. Partition coefficient (logP) obtained by means of different theoretical methods and by use of shake flask method in *n*-octanol - water system ($logP_{exp}$).

Partition coefficient	
Taken from online software package	
$logP_{exp}$	2.78
AlogPs	3.10
AClogP	2.98
AlogP	3.59
MlogP	3.77
$logP_{KOWWIN}$	2.88
xlogP2	3.41
xlogP3	2.93
$logP_{average}$	3.24 (± 0.36)
Calculated on the basis of topological indices	
$logP_1$	2.73
$logP_2$	3.24
$logP_3$	3.00

where: logk is the logk value of spironolactone, $logk_w$ is the logk value of spironolactone extrapolated to zero concentration of organic modifier in mobile phase, S is the slope of the regression plot, φ is the volume fraction of organic modifier in applied mobile phase.

Calculations of partition coefficients by computational methods

Theoretical partition coefficients of spironolactone, such as AlogPs, $logP_{KOWWIN}$, xlogP2, xlogP3, AClogP, AlogP, MlogP and also average value of logP, which have been predicted on the basis of chemical structure of investigated compound by means of various computational procedures, were obtained from drugbank and also from another database available *via* online at VCCLAB.org website (36, 37). All theoretically determined partition coefficients and also the *n*-octanol partition coefficient ($logP_{exp}$) predicted by the use of classical shake flask method taken from VCCLAB.org website are presented in Table 1.

Newly developed method of calculation of logP

In order to calculate the logP value the selected topological indices based on adjacency matrix: Gutman (M^v and M), Randic ($^0\chi^v$, $^0\chi$ and $^1\chi$) and also based on distance matrix: Pyka (^0B, ^1B), Wiener (W), and Balaban (I_B) were calculated. The numerical values of calculated topological indices are listed in Table 2. The method of calculation of these indices have been described elsewhere (38-41). Topological indices based on distance matrix were calculated by building a distance matrix and by determining its elements by means of values given by Barysz et al. (42).

Table 2. Numerical values of the selected topological indices calculated for examined spironolactone.

The topological indices based on:	
Adjacency matrix	
M	216.000
$^0\chi$	10.286
$^1\chi$	12.384
M^v	340.440
$^0\chi^v$	18.608
Distance matrix	
W	1678
^0B	2.7288
^1B	0.3079
I_B	1.6936

The proposed new methods of calculation of logP value denoted as $logP_1$, $logP_2$ and $logP_3$ for examined compound based on its topological indices are characterized by the following formulae: [5], [6] and [7]

$$logP_1 = {}^0B \quad [5]$$

$$logP_2 = \frac{W}{M^v} - I_B \quad [6]$$

$$logP_3 = {}^0\chi^v \cdot {}^1B - {}^0B \quad [7]$$

where: , ^0B, ^1B, W, $^0\chi^v$ and I_B are topological indices.

Regression and cluster analysis

Regression and cluster analysis of obtained results were performed with the use of computer software STATISTICA 10.0.

RESULTS AND DISCUSSION

This work is a part of our previous study on lipophilicity determination of biologically active steroids. Recently, we have estimated the applicability of reversed phase thin-layer chromatography (RP-TLC and RP-HPTLC) as well as computational methods to describe the lipophilicity of selected bile acids, some steroid anabolics, plant sterols (26-34) and non-steroidal compounds namely salicylic and acetylsalicylic acids (35). Numerous chromatographic systems were applied in order to determine lipophilicity descriptor (R_{MW}) for examined steroids which have shown various pharmacological action. Our investigations confirmed that the experimentally determined by thin-layer chromatography lipophilicity parameter (R_{MW}) and some computed logP (calculated by use of appropriate programs) may be used as alternative to *n*-octanol - water partition coefficient in describing lipophilic properties of steroids. Besides obtaining reliable lipophilicity descriptor of previously tested steroid compounds (e.g., bile acids), the advantage of the proposed TLC method was a possibility of examination of several discussed steroids such as bile acids in parallel stage (on the same chromatographic plate). According to our knowledge, until today, there is no paper containing a comparative study of the chromatographically (by TLC and HPLC) and also computed determined lipophilicity descriptors of spironolactone.

Therefore, the present lipophilicity study is a continuation of those earlier reported, in which comparison of different lipophilicity descriptors determined by use of chromatographic methods: RP-TLC, RP-HPTLC, RP-HPLC and also those calculated including the newly developed based on

numerical values of topological indices for spironolactone was performed. Application of the numerical values of selected topological indices, such as M^v, ^0B, ^1B, W, $^0\chi^v$ and I_B to calculate the partition coefficients: $logP_1$, $logP_2$ and $logP_3$ of tested spironolactone was the novelty of this study.

In order to determine and then to estimate the compatibility of the chromatographic lipophilicity parameter (R_{MW}) determined by reversed phase TLC with those calculated by means of RP-HPLC and also obtained by use of other procedures, e.g., theoretical and with *n*-octanol - water partition coefficient $logP_{exp}$ (obtained by shake flask method), regardless of the applied chromatographic systems, extrapolation of R_M values to zero content of organic modifier φ (methanol, acetone, dioxane) in mobile phase according to Eq. 2 was done. Parameters of linear relationships between R_M and φ such as *r* - correlation coefficient, *s* - standard error, p - significance level and *F* value of Fischer test are listed in Table 3. Analysis of correlation coefficients in Table 3 (*r* above 0.9) indicates that strong correlations were obtained for all modifiers in the range of 55-90%. Thus, all discussed linear dependences may be satisfactory applied to determine relative lipophilicity parameter R_{MW} for tested compound. The results of R_{MW} (± SD) obtained under 9 chromatographic systems: on different chromatographic plates and by various mobile phases are presented in Table 3. From the data presented there, it could be concluded that obtained R_{MW} values are placed in the range of: 2.513 - 3.476. In order to estimate the impact of organic modifier of all applied (methanol, acetone and dioxane) on chromatographic retention of tested compound, the R_{MW} values determined using RP-TLC and RP-HPTLC plates and by use of these three mobile phase systems were compared using cluster analysis (single-bond method, *Euclidean-distance*) in Figure 1. Dendrogram (see Fig. 1) indicates a big similarity between R_{MW} values determined on all applied chromatographic plates used in this experiment which have been developed with the use of dioxane - water ($R_{MW(d)}$) and also acetone - water ($R_{MW(a)}$) as the mobile phases. This fact could be explained by similar behavior of spironolactone developed in both solvent systems. Thus, it

Table 3. Parameters of Eq. [2] (RP-TLC and RP-HPTLC) and Eq. [4] (RP-HPLC) calculated for tested compound.

Parameters of linear correlations $R_M=R_{MW}-S\times\varphi^*$						
Stationary phase type	R_{MW}(±SD)	S(±SD)	r	s	F	n
methanol – water (v/v)						
Silica gel RP-18WF_{254}	3.476 (± 0.229)	4.593 (± 0.322)	0.983	0.124	203.8	9
Silica gel RP-18F_{254}	3.474 (± 0.243)	4.264 (± 0.319)	0.986	0.080	178.4	7
Silica gel RP-2F_{254}	2.942 (± 0.080)	4.283 (± 0.113)	0.998	0.044	1442.1	9
acetone – water (v/v)						
Silica gel RP-18WF_{254}	2.564 (± 0.182)	3.756 (± 0.261)	0.988	0.092	206.9	7
Silica gel RP-18F_{254}	3.035 (± 0.190)	4.064 (± 0.272)	0.989	0.095	223.8	7
Silica gel RP-2F_{254}	2.513 (± 0.220)	3.804 (± 0.316)	0.983	0.011	145.2	7
dioxane – water (v/v)						
Silica gel RP-18WF_{254}	2.527 (± 0.162)	3.931 (± 0.247)	0.988	0.106	254.2	7
Silica gel RP-18F_{254}	2.855 (± 0.103)	4.238 (± 0.155)	0.997	0.066	746,0	8
Silica gel RP-2F_{254}	2.778 (± 0.133)	4.464 (± 0.203)	0.994	0.087	481.8	8
Parameters of linear correlations (± SD) $logk = logk_w - S \times \varphi^*$						
Stationary phase type	$logk_w$	S	r	s	F	n
methanol – water (v/v)						
Silica gel RP-18	3.144 (± 0.030)	3.921 (± 0.040)	0.999	0.020	8112.0	9
dioxane – water (v/v)						
Silica gel RP-18	1.952 (± 0.124)	3.193 (± 0.163)	0.994	0.053	382.9	6

Notes: *n*-number of points used to derive the particular regressions; *r*-correlation coefficient; *s*- standard error; *F*-value of Fischer test; * for all equations the significance level $p < 0.001$.

could be suggested that acetone may be applied alternatively to dioxane as mobile phase component in lipophilicity study of examined compound.

In further investigations, to estimate utility of high-performance RP-HPLC to predict the lipophilicity parameter of spironolactone expressed as $logk_w$, the logk values obtained on the basis of t_R values (Eq. 3) were extrapolated to zero content of organic modifier (methanol or dioxane) in applied mobile phases: methanol - water and dioxane – water, respectively, in accordance with Eq. 4. Exemplary TLC and HPLC chromatograms of spironolactone obtained with methanol - water systems are shown in Figure 2 A and B, respectively. Parameters of linear correlations between logk and j, such as *r*-correlation coefficient, *s*-standard error, p-significance level and *F* value of Fischer test are presented in Table 3. Satisfactory results of *r* values which range from 0.994 to 0.999 show that the obtained linear correlations between logk and φ allow to determine relative lipophilicity descriptor expressed in HPLC as $logk_w$. It can be observed that $logk_w$ predicted by use of dioxane - water ($logk_{w(d)}$) is visibly lower compared to those predicted with the use of methanol - water ($logk_{w(m)}$). Obtained $logk_w$ was 1.952 in the case of used dioxane as organic modifier of mobile phase and 3.144 for methanol, respectively (Table 3). We have disqualified in HPLC lipophilicity measurements the third of applied organic modifier - acetone, due to observed inacceptable results of t_R caused by irregular noise baseline on chromatograms of spironolactone detected using mobile phase acetone - water. This fact confirmed the suggestion performed by Komsta et al. (16) that of several modifiers, methanol and dioxane are the best in lipophilicity determination by use of TLC and HPLC methods.

Partition coefficients logP calculated by means of software packages available online (at the Virtual Computational Chemistry Laboratory) and also experimental logP (determined by shake flask method) summarized in Table 1 indicate certain discrepancies among themselves. Generally, computed logP which has been predicted by various algorithms is placed in the range: from 2.88 ($logP_{KOWWIN}$) to 3.77 (MlogP). Thus, average value of partition coefficient calculated on the basis of all computed logP is 3.24 (± 0.36). As it can be seen, this value is relatively higher in relation to *n*-octanol - water partition coefficient ($logP_{exp}$) obtained from available software. Among all computationally calculated logP the most similar to $logP_{exp}$ is $logP_{KOWWIN}$ and $xlogP_3$. In addition to this, the greatest similarity to average logP indicates AlogPs and also xlogP2. These results indicate that in order to apply the computed logP as a measure of lipophilicity of examined compound, critical review of all available logP for spironolactone should be done.

Analysis of partition coefficients obtained by the use of proposed procedure based on topological indices (Table 1) demonstrates that the first method of calculations (based on topological index ^0B, Eq.

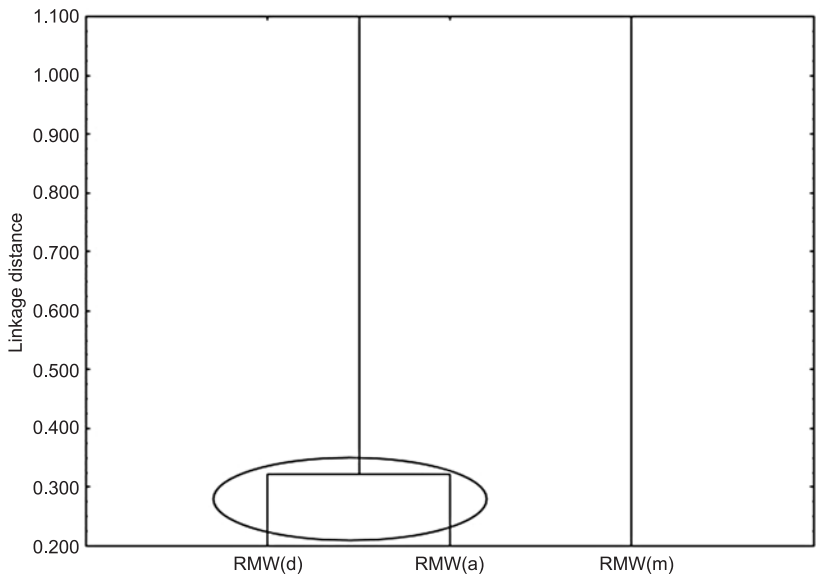

Figure 1. Cluster analysis of lipophilicity descriptors (R_{MW}) determined for spironolactone by means of RP-TLC and RP-HPTLC and binary solvent systems: methanol – water (m); acetone – water (a) and dioxane – water (d) as mobile phases

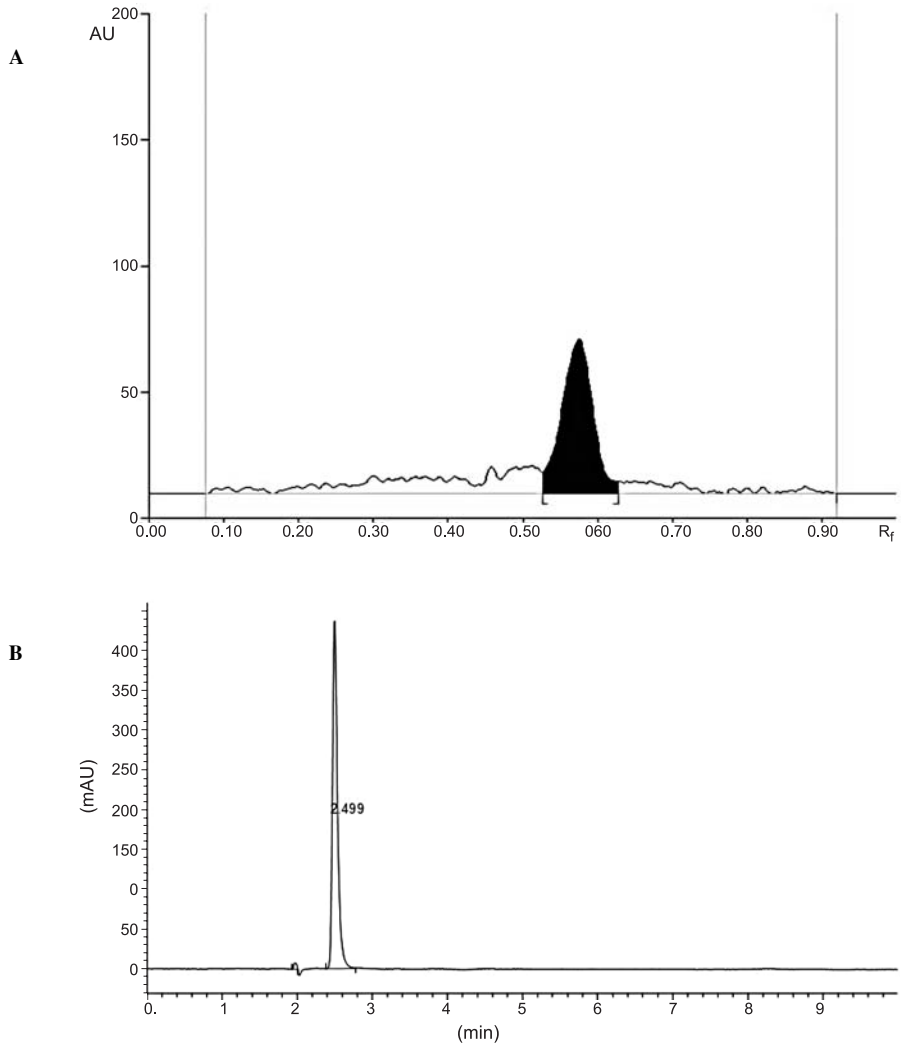

Figure 2. Exemplary TLC chromatogram of examined spironolactone obtained on chromatographic plates RP-18F_{254} using methanol – water: 90 : 10 (v/v) (**A**); example of HPLC chromatogram of spironolactone investigated on column RP18 by the use of methanol - water in volume composition: 90 : 10 (v/v) (**B**)

5) gives the result of lipophilicity descriptor (designated as $logP_1$) very similar to $logP_{exp}$. Other partition coefficients calculated by means of various topological indices according to Eq. 6 and Eq. 7 presented in Table 1 are $logP_2$ and $logP_3$ which show higher similarity to $logP_{average}$. It could be suggested that topological indices may be useful in predication of lipophilic properties of steroid compounds like, for example, spironolactone.

As it was accurately emphasized in introduction part of this work, in order to estimate which of the theoretically determined (by different calculations procedures) partition coefficient may be a reliable measure of lipophilicity of examined biomolecule there is a need to compare all calculated logP values with those obtained by appropriate experimental method. Therefore, the third stage of this study was the comparison and assessment of all obtained results. Compared experimental and calculated lipophilicity descriptors for spironolactone are presented in Figure 3.

As results from Figure 3, of all chromatographically determined lipophilicity descriptors (R_{MW}) the biggest similarity to $logP_{exp}$ shows R_{MW} obtained on glass RP-HPTLC plates RP-18F_{254} and RP-2F_{254} developed with mobile phase: dioxane - water: $RMWRP18_{(d)}$ and also $RMWRP2_{(d)}$. Among computed partition coefficients, these which are comparable to those are $logP_{KOWWIN}$ and $xlogP_3$. The results of RP-HPLC analysis indicate that the chro-

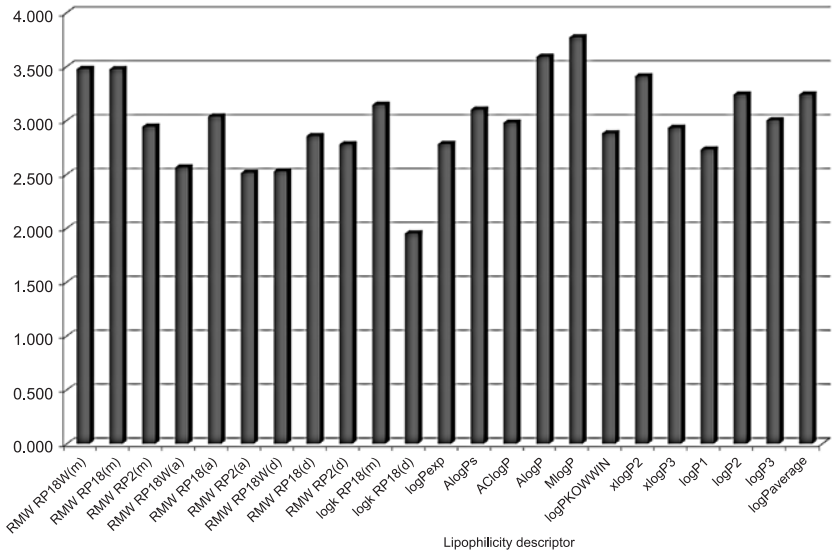

Figure 3. Comparison of experimental and calculated lipophilicity descriptors for spironolactone determined using various methods: (m) methanol - water; (a) acetone - water and (d) dioxane - water,; $logP_{exp}$ – the experimental partition coefficient determined by shake flask method; $logP_1$, $logP_2$ and $logP_3$ – partition coefficients calculated on the basis of topological indices

matographic parameter of lipophilicity predicted by this technique in methanol - water system and denoted as $logkRP18_{(m)}$ is relatively higher in relation to $logP_{exp}$ but correlates well with the computed logP, AlogPs and also with average value of logP ($logP_{average}$). The second parameter estimated by means of RP-HPLC and dioxane as organic modifier of mobile phase – $logkRP18_{(d)}$ demonstrates much lower value (about 2) in comparison with other lipophilicity parameters. Thus, no significant relation between this parameter and also others obtained in this study was observed. It confirms previous suggestion that dioxane gives better results of lipophilicity measurements conducted by TLC than by HPLC. The last group of estimated lipophilicity parameters of examined spironolactone are those, which have been calculated on the basis of the numerical values of selected topological indices based on adjacency and on distance matrix, respectively: M^v, ^0B, ^1B, W, $^0\chi^v$ and I_B according to the proposed formulae (Eq. 5-7). These partition coefficients: $logP_1$, $logP_2$ and $logP_3$ are placed in the range of: 2.73-3.24. Among them, $logP_1$ based on topological index ^0B is in good agreement with $logP_{exp}$ and also with chromatographically predicted lipophilicity parameter R_{MW} in dioxane - water system on RP-$2F_{254}$ plates (RMWRP2(d)). Good correlation could be observed also between $logP_1$ and the following computationally determined partition coefficients: AClogP, $logP_{KOWWIN}$ and $xlogP_3$.

Next partition coefficient determined by newly developed procedure based on topological indices: W, M^v, and I_B described by Eq. 6 enabled calculate $logP_2$ which shows the biggest similarity to chromatographic parameter of lipophilicity $logk_w$ determined by use of methanol - water (logkRP18(m)) and also with computationally determined $logP_{average}$. The third developed partition coefficient ($logP_3$) indicates the biggest similarity to the R_{MW} obtained on RP-$2F_{254}$ plates developed with mobile phase: methanol - water and also on silica gel RP-$18F_{254}$ using acetone - water as the mobile phase.

Finally, it can be concluded, that the results of lipophilicity parameters of spironolactone obtained by the use of TLC and HPLC indicate that liquid chromatography can play important role as an experimental method in lipophilicity study of certain steroids like spironolactone because is accurate, not expensive and does not require a large amount of compound in comparison with classical shake flask method. Additionally, it has been stated that the best (optimal) chromatographic conditions which allowed obtain the lipophilicity results (expressed as R_{MW} and $logk_w$) similar to those determined by the use of reference shake flask method are: dioxane – water and silica gel RP-$2F_{254}$ and RP-$18F_{254}$ in the case of TLC. In the case of HPLC a mixture of methanol - water (as mobile phase) and column RP18 (as the stationary phase) are optimal in lipophilicity study of spironolactone.

Further investigations will be continued. The predicted by different theoretical methods and also chromatographically determined lipophilicity descriptors of spironolactone will be applied not for description of its lipophilicity only but also to estimate the efficiency and applicability of newly developed logP calculation models based on topological indices to evaluate the pharmacokinetic properties of tested spironolactone and its metabolite like canrenone in future QSAR study.

CONCLUSIONS

From the analysis of obtained data, it can be concluded that:

- liquid chromatography in reversed-phase system, such as RP-TLC, RP-HPTLC and also RP-HPLC can be an alternative method to traditional shake flask procedure for studying lipophilicity of spironolactone;
- R_{MW} and $logk_w$ parameters can be used as an estimation of the lipophilicity of spironolactone;
- partition coefficients logP calculated according to molecular structure of tested compound by use of online available package software, such as AlogPs, $logP_{KOWWIN}$, xlogP2, xlogP3, AClogP, AlogP and MlogP demonstrate certain discrepancies which could be explained by differences in accuracy of these calculations;
- newly developed logP calculation models based on topological indices are suitable for predication of partition coefficients of investigated spironolactone denoted as $logP_1$, $logP_2$ and $logP_3$, respectively;
- among performed theoretical lipophilicity parameters, those which are comparable with *n*-octanol - water partition coefficient ($logP_{exp}$) determined by shake flask method are: computed $logP_{KOWWIN}$, xlogP3 and also the newly developed $logP_1$;
- of all chromatographic lipophilicity descriptors: R_{MW} and $logk_w$ those which correlate well with $logP_{exp}$ are R_{MW} values determined by use of dioxane - water system and silica gel (RP-$2F_{254}$ and RP-$18F_{254}$);
- obtained lipophilicity parameters including chromatographic results, such as R_{MW} and $logk_w$ can be used in future QSAR study of spironolactone and its metabolite like canrenone.

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DRUG BIOCHEMISTRY

ANTIOXIDANT ACTIVITY AND FREE RADICAL SCAVENGING CAPACITY OF L-ARGININE AND NAHS: A COMPARATIVE *IN VITRO* STUDY

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Abstract: In the family of gaseous transmitters, hydrogen sulfide (H_2S) is considered as third member beside nitric oxide (NO) and carbon monoxide (CO), which can play physiological role in different organs. The present study was designed to elucidate the antioxidant and free radical scavenging potentials of L-arginnine (a source for endogenous production of NO *in vivo*) and NaHS (a source H_2S) individually and in combination. Different assays like 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging, percent inhibition of linoleic acid peroxidation and reducing power assays were used to evaluate the free radical scavenging capacity and antioxidant activity of L-arginine and NaHS. Furthermore, study was aimed to know the antioxidant potential of both compounds at their effective doses in human body, which is 56 μM for H_2S and 1.2 g/mL for L-arginine. The study also aimed to clear whether either NaHS, L-arginine or the mixture of NaHS and L-arginine *in vitro* (in the form of new compounds) is responsible for their therapeutic action. Results showed that NaHS, L-arginine and combination of NaHS + L-arginine showed good radical scavenging activity i.e., 55.60%, 52.10% and 52.32%, respectively. Moreover, NaHS was found to have ability to inhibit linoleic acid peroxidation by 53.98% at effective dose while L-arginine did not show inhibition of linoleic acid peroxidation. Combination of NaHS + L-arginine showed 54.15% inhibition of linoleic acid peroxidation, which is similar to that of H_2S. Reducing power of NaHS was 0.073 and L-arginine showed 0.037, combination of NaHS + L-arginine showed 0.063. It can be concluded that NaHS showed better antioxidant potential *in vitro* as compared to L-arginine and the antioxidant activity of the mixture of NaHS + L-arginine is closed to the antioxidant activity of NaHS, which reflects that NaHS is a dominant factor in combination mixture that is responsible for antioxidant activity.

Keywords: H_2S, NO, DPPH, linoleic acid peroxidation

Imbalance between prooxidant and antioxidant activity in human body results in the development of oxidative stress, which is considered as a major route causing cardiovascular disease, cancer (1), hypertension (2), acute respiratory distress syndrome (3), chronic inflammatory diseases (4), ischemia/reperfusion injury (I/R) (5), Parkinson's disease and Alzheimer's disease (6) as well as aging (7). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) start accelerate prooxidant processes and produce toxic results in the body. Hence, there is a need of antioxidants which scavenge these free radicals and makes them inactivate.

Medical gases including hydrogen sulfide (H_2S) and nitric oxide (NO) and carbon monoxide (CO) have captured the interest of researchers by their number of applications in human body. Endogenous H_2S is produced from two sulfur containing amino acids - L-cysteine and L-methionine, by the two enzymes cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS) (8). The endogenous concentration of circulating H_2S is 50–160 μM in rat, bovine and human (9). NaHS have been used previously as a donor of H_2S (10, 11). H_2S is found to have many therapeutic applications like anti-inflammatory activity (12) and is involved in regu-

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lation of liver and gastrointestinal functions (13). H_2S can effectively prevent hypertension in rats if it is induced by N^G-nitro-L-arginine methyl ester (L-NAME) (14). Nitric oxide produces vasodilatation by activating cyclic guanosine monophosphate (cGMP) on blood vessels. NO, which is produced from endothelial NOS, reduces the contractility of heart by inhibiting the influx of Ca^{2+} (15). So, systemic or endogenous production of NO in heart failure case can provide protection by its vasodilator and negative ionotropic response. In support of this statement, it is evident that low dose of β blockers (especially carvedilol) have been found to have protective role in case of heart failure by decreasing heart rate (16). L-arginine has been used as precursor of NO (17, 18). L-arginine undergoes enzymatic reaction resulting in endogenous production of NO with the help of endothelial nitric oxide synthase (eNOS). It is evident that L-arginine enhances the antioxidant activity of garlic (19). NaHS has sulfur content like garlic so it is expected from L-arginine to enhance the antioxidant activity of NaHS.

Both medical gases have captured the interest of researchers since last decades but still many therapeutic applications needs clarity like interdependable production of H_2S and NO or an intermediate molecule formation (11, 20, 21). Present study aimed to solve this mystery by using *in vitro* study first, without enzymatic involvement. After these experiments, will be conducted *in vivo* studies for enzymatic action and outcome correlation with *in vitro* study. This study was conducted to evaluate the *in vitro* antioxidant potential of NaHS and L-arginine alone and in combined form. Furthermore, this study was extended to answer the dispute between different schools of thought whether an intermediate product is formed by combining NaHS and L-arginine or anyone of these two is dominant in this mixture. This factor will be partially studied in presented *in vitro* studies.

MATERIALS AND METHODS

Chemicals

L-arginine as a source of nitric oxide (NO) and NaHS as a source of H_2S, linoleic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and butylated hydroxytoluene (BHT) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other chemicals of analytical grade like anhydrous sodium carbonate, ammonium thiocyanate, ferrous chloride ($FeCl_2$), methanol and chloroform were purchased from Merck (Darmstadt, Germany).

Preparation of solutions

Solutions were prepared on the basis of ED_{50} values already reported in previous studies. First of all, individual solutions of NaHS (3.125–100 µM) and L-arginine (0.075–2.4 mg/mL) were prepared as shown in Table 1. Combined solution of NaHS and L-arginine was prepared in such a way that effective doses (ED_{50}) or therapeutically active concentrations of NaHS and L-arginine were mixed together as shown in Table 1.

Antioxidant activity

Antioxidant activities of abovementioned 3 forms of drugs were investigated using the following methods.

DPPH radical-scavenging activity

Antioxidant activities of NaHS, L-arginine and combination of NaHS + L-arginine were assessed by their ability to scavenge free stable radicals like 2,2-diphenyl-1-picrylhydrazyl (DPPH). The assay was performed as reported before (22). Different concentrations of sodium hydrogen sulfide (NaHS) (3.125–100 µM) and L-arginine (0.075–2.4 mg/mL) were prepared on the basis of their doses used for different studies. A third solution was prepared by combining the serial dilution

Table 1. Solutions of NaHS, L-arginine and their mixture used in the study.

Conc.	NaHS (µM)	L-arginine (mg/mL)	NaHS + L-arginine (µM + mg/mL)
F	100	2.4	100 + 2.4
E	50	1.2	50 + 1.2
D	25	0.6	25 + 0.6
C	12.5	0.3	12.5 + 0.3
B	6.25	0.15	6.25 + 0.15
A	3.125	0.075	3.125 + 0.075

in such a manner that the effective doses of NaHS + L-arginine (50 µM + 1.2 mg/mL) were mixed together as shown in Table 1.

Samples (125 µL) were mixed with 125 µL of 90 µM solution of DPPH in methanol. BHT was taken as a positive control. The samples were incubated at room temperature for 30 min and the absorbance was recorded at 515 nm using spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Percentage (%) scavenging of DPPH free radical was calculated by the following formula:

$$RS\ (\%) = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

where A_{blank} is the absorbance of the control whereas A_{sample} is the absorbance of the tested samples.

Percentage inhibition of linoleic acid peroxidation for NAHS, L-arginine and combination of NaHS + L-arginine

Linoleic acid peroxidation inhibition was done by using method described in (23). Different concentrations of NaHS, L-arginine and combination of NaHS + L-arginine were used as shown in Table 1. Samples (5 mg) of NaHS, L-arginine and combination of NaHS + L-arginine were mixed with 0.13 mL of linoleic acid, 10 mL of 99.8% ethanol and 10 mL of 0.2 M sodium phosphate buffer (pH = 7). The mixture was diluted with water up to 25 mL and the solution was incubated for 175 h at 40°C in an incubator. The extent of oxidation was measured by colorimetric method (24). BHT was taken as a positive control and sample without antioxidant is taken as blank.

Percentage inhibition of linoleic acid peroxidation was calculated by using the following formula: 100 – [(Abs. increase of sample after incubation / Abs. increase of control after incubation) × 100].

Reducing power assay for NaHS, L-arginine and combination of NaHS + L-arginine

Reducing power assay was performed as described in (23). Various concentrations of NaHS, L-arginine and NaHS + L-arginine were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide. These mixtures were incubated at 50°C for 20 min. After this, 2.5 mL of 10% trichloroacetic acid were added and the mixture was centrifuged at 650 rpm for 10 min. The upper layer was mixed with 5 mL of deionized water and 1 mL of 0.1% ferric chloride and absorbance was measured at 700 nm. High absorbance indicates high reducing power. BHA was used as a positive control.

RESULTS AND DISCUSSION

The endogenous concentration of circulating H_2S is 50–160 µM in rat, bovine and human (22, 23).Therapeutic dose of H_2S considered for animal is 56 µM as the vasorelaxant effects of H_2S has been proved by (24), which shows that H_2S relaxes the isolated aorta at concentration as low as 18 µM and 60 µM pretreated with 20 mM KCl or PHE, respectively. Antioxidant role of sodium hydrogen sulfide has been previously reported partially (11) as scavenger (21) protecting neurons against oxidative stress (25). In one study (24) it was demonstrated that plasma level of H_2S was 50 µM. Tissue level of H_2S is thought to be higher than plasma level. So, therapeutic concentration of H_2S is considered to be 56 µM.

DPPH radical-scavenging activity

NaHS is used in different concentrations ranging from 3.125 to 100 µM as shown in Figure 1.

Free radical scavenging activity of NaHS, L-arginine and equimolar concentrations of both NaHS and NO were evaluated by their ability to scavenge DPPH free radical. Abovementioned figure shows that the lowest concentration of NaHS, which is 3.125 µM, has 47.04% scavenging ability whereas 50 µM has 55.60% and maximal dose (100 µM) has maximum scavenging 75.84%. So, maximum concentration of NaHS has the highest antioxidant potential. Therapeutic dose (56 µM) gives 61.39% scavenging of DPPH and is showing good antioxidant potential which is ideal from safety and efficacy point of view. These results show that NaHS has antioxidant activity by scavenging free radicals and these *in vitro* results justify the use of NaHS *in vivo* as well.

L-arginine is a precursor of nitric oxide (NO). NO is generally known as prooxidant and inflammatory mediator (26, 27). L-arginine is used in different concentrations ranging from 0.075 mg/dL to 2.4 mg/dL. In order to make comparison with NaHS, L-arginine solution has been used in therapeutic range which is 1.2 mg/dL (27). Radical scavenging potential of different concentration of L-arginine is shown in Figure 2. Therapeutic dose (1.2 mg/dL) has shown 52.10% free radical scavenging, which is higher than for minimum concentration of L-arginine (0.075 mg/dL) with 46.83% free radical scavenging and less than that of maximum concentration (2.4 mg/dL) with 63.08% free radical scavenging. Scavenging potential of L-arginine has been proved previously (27) but no other mechanisms were elucidated to prove it as antioxidant.

Proposed mechanism for NO may be as below:

$$\text{Lipid} \xrightarrow{O_2} \text{LOO}^{\bullet} \xrightarrow{NO^{\bullet}} \text{LOONO} \begin{cases} \xrightarrow{NO^{\bullet}} \text{LONOO} \\ \text{LONO/LONOO} \\ \text{LOOH} + NO_2 \end{cases}$$

L = lipid

Above highlighted reaction is rate limiting step and calculation suggested that in *in vivo* settings, NO is better scavenger of LOO as compared to tocopherol. This reaction also showed that 2 molecules of NO are consumed for each LOO molecules but rate of reaction is faster than tocopherol potential antioxidant (27).

Scavenging potential of NO depicts its antioxidant potential by scavenging free radicals.

When NO and H_2S are produced inside the body by using their precursors, an intermediate compound is formed that may be nitroxyl or nitrosothiol (11, 20, 21, 24, 26). Present study was aimed to know the difference in antioxidant potential of donors of H_2S and NO alone and in combination. These finding may serve baseline studies in *in vivo* models of study. In this *in vitro* assay 3 solutions were prepared NaHS, L-arginine and combination of NaHS and L-arginine as shown in Table 1. Combined solution was prepared in such a way that therapeutic concentrations of NaHS and L-arginine were in the same concentration in mixture (that is E).

Results of DPPH free radical scavenging assay showed that NAHS, L-arginine and combination of NaHS and L-arginine at doses (50 µM, 1.2 mg/mL and 50 µM + 1.2 mg/mL) showed 55.61%, 52.1% and 52.32 % scavenging of free radicals at therapeutic doses, respectively, as shown in Figure 3. At maximum doses of NAHS, L-arginine and combination of NaHS and L-arginine (100 µM, 2.4 mg/mL

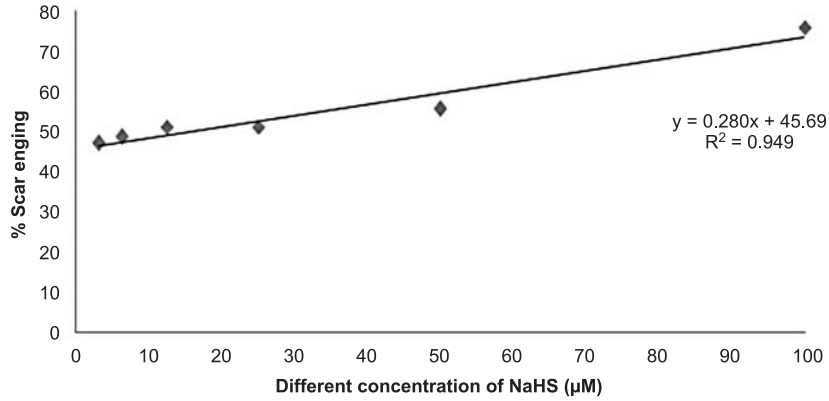

Figure 1. Antioxidant activity of different concentrations of NaHS

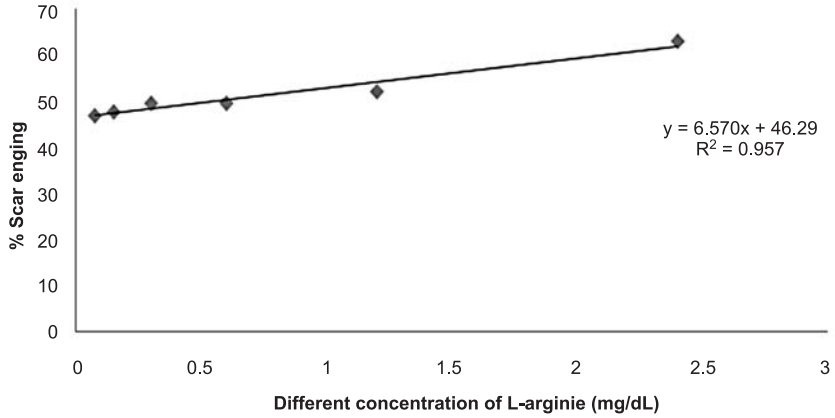

Figure 2. Antioxidant potential of different concentrations of L-arginine

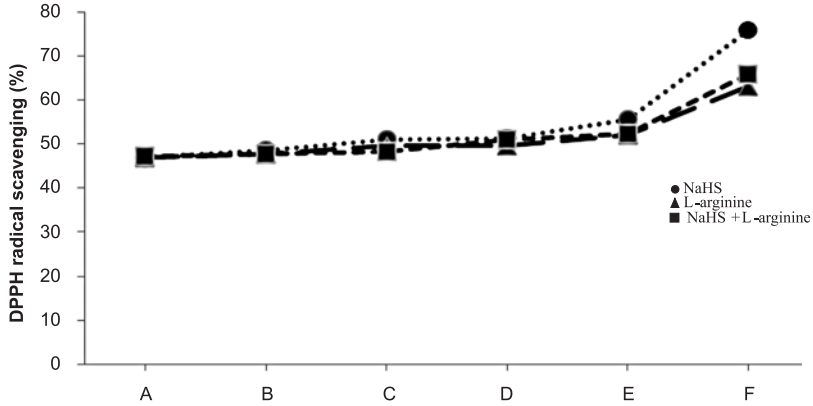

Figure 3. Antioxidant potential comparison of NaHS, L-arginine and combination of equimolar concentration of (NaHS + L-arginine) showing scavenging ability of different concentrations of NaHS (cf. Table 1) in DPPH assay

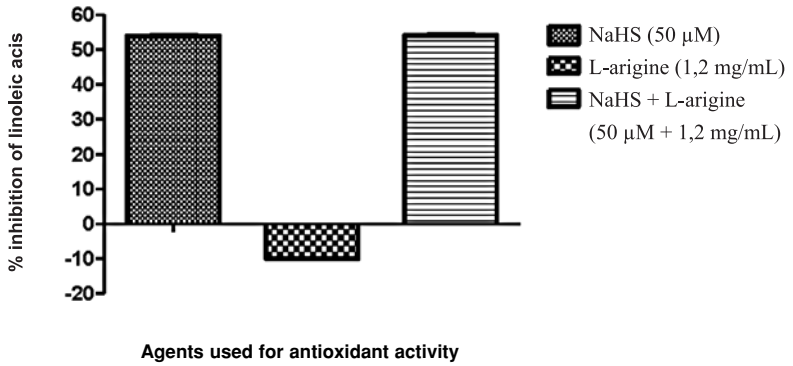

Figure 4. Comparison between NaHS, L-arginine and NaHS + L-arginine in linoleic acid peroxidation inhibition

and 100 µM + 2.4 mg/mL) free radical scavenging was 75.84, 63.08 and 65.82%, respectively. At minimum doses, 3.125 µM, 0.075 mg/mL and 3.125 µM + 0.075 mg/mL of NaHS, L-arginine and NaHS + L-arginine, they showed 47.04, 46.83 and 47.15% of free radical scavenging activity, respectively. Free radical scavenging abilities of NaHS, L-arginine and combination of both showed similar results. These *in vitro* results confirm and justify the *in vivo* use of these drugs to validate antioxidant potential.

Linoleic acid peroxidation inhibition by NAHS, L-arginine and combination of NAHS + L-arginine

Inhibition of linoleic acid peroxidation is another role of any antioxidant to play, so NAHS, L-arginine and NAHS + L-arginine were tested for their ability to inhibit linoleic acid peroxidation.

NaHS showed linoleic acid peroxidation inhibition by 53.98% at therapeutic concentration (50 µM) and L-arginine showed no inhibition of linoleic acid peroxidation even at therapeutic dose (1.2 mg/mL). However, combination of both doses of NaHS and L-arginine showed linoleic acid peroxidation inhibition by 54.15%, which is similar to that by H_2S. It appears from the data that L-arginine does not inhibit linoleic acid peroxidation, so NaHS is playing predominant role in this mechanism. In combined solution, linoleic acid peroxidation inhibition may be due to NaHS as percentage of inhibition is very close to that of NaHS or may be due to some intermediate compound that is formed by mixing both solutions.

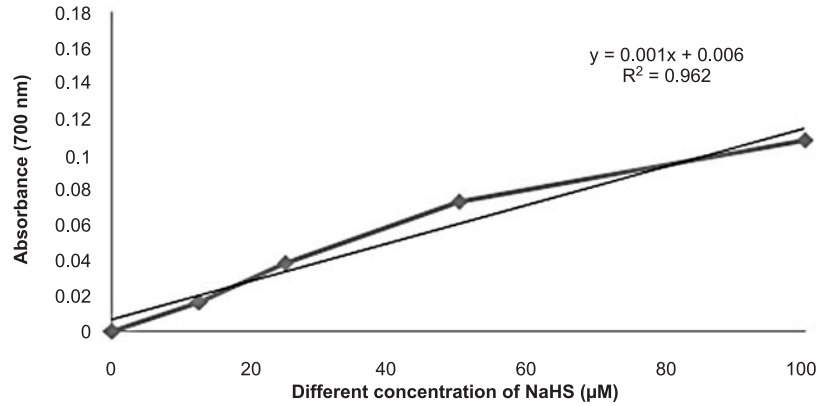

Figure 5. Reducing power potential of NaHS at different concentrations

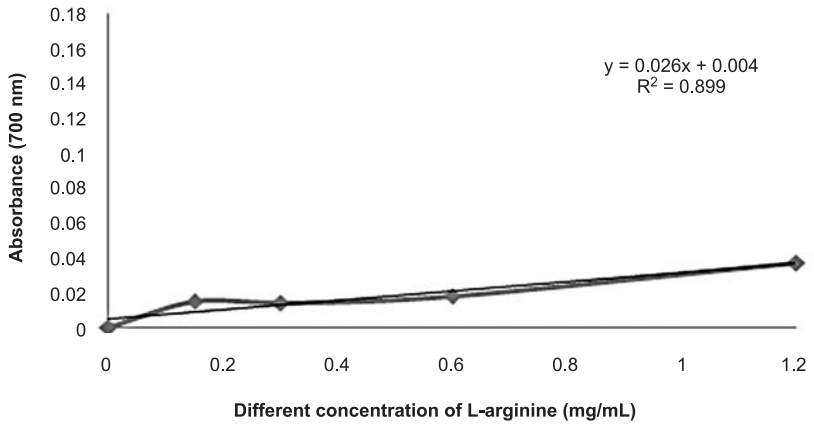

Figure 6. Reducing power potential of L-arginine at different concentrations

Reducing power assay by NaHS, L-arginine and combination of NaHS + L-arginine

Figure 5 is showing the reducing power potential of NaHS at different concentrations. Chemistry of this assay involves the conversion of yellow color to various shades of blue to green color. The presence of antioxidant which is reducing agent in its potential, changes ferric/ferricyanide to ferrous form Fe^{2+}. From the figure it is clear that NaHS has reducing power ability from 0–100 µM concentration. Present study measure the reducing power potential by following procedure which was reported earlier (27). The greater the absorbance the higher will be reducing power ability. Reducing power of NaHS at therapeutic dose is 0.33 at 700 nm while response remains the same when the concentration was enhanced up to 100 µM.

Nitric oxide being prooxidant has shown weak reducing power ability which is one of the factors contributing to its antioxidant potential. Not being potent reducer, L-arginine (precursor of NO) showed its weak reducing ability in the concentration ranging between 0 to 1.2 mg/mL as shown in Figure 6. Therapeutic dose (1.2 mg/mL) showed absorbance 0.030 at 700 nm.

When combined solution of NaHS + L-arginine was tested for reducing ability, the results were more similar with that of NaHS as shown in Figure 7, which suggests that in this mixture either it is NaHS playing dominant role or it is due to an intermediate product that is contributing to its role. Minimum dose A of combined solution showed no activity while dose F has shown significantly the highest absorbance 0.16 as compared to 0.10 of

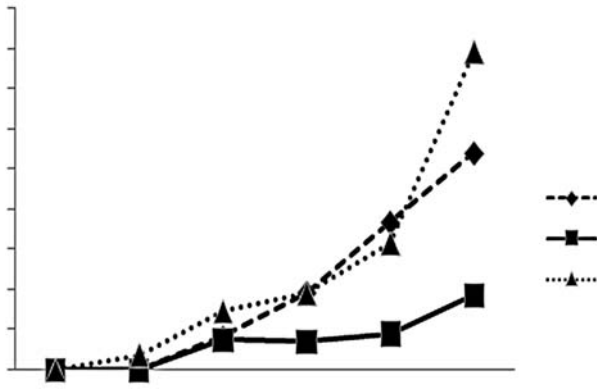

Figure 7. Comparison of reducing power assay between NaHS, L-arginine and NaHS + L-arginine

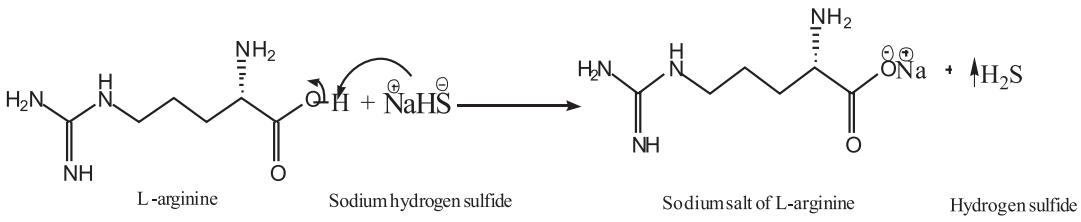

Figure 8. Proposed mechanism of *in vitro* reaction between NaHS and L-arginine showing the release of H_2S.

NAHS and 0.03 of L-arginine. Concentration E (1.2 mg/mL of L-arginine and 50 μM NaHS) showed better response (0.16) as compared to other concentrations.

Proposed mechanism of NaHS + L-arginine

When NaHS is combined with L-arginine, an acid base reaction will take place as shown, as bisulfide is a strong base so it will abstract a proton from L-arginine resulting in the formation of its sodium salt along with evolution of hydrogen sulfide gas. Apparently, it can be concluded that both NaHS and L-arginine in combined solution individually produced their pharmacological responses as shown in the proposed mechanism (Fig. 8). However, *in vivo* study may solve this ambiguity.

CONCLUSION

Present study has demonstrated that sodium hydrogen sulfide has potential antioxidant activity by free radical scavenging, inhibiting linoleic acid peroxidation and as a reducing agent. L-arginine showed weak antioxidant activity by reducing power assay but is good free radical scavenger. In comparison, NaHS is more potent antioxidant than L-arginine, whereas on combination, reaction between NaHS and L-arginine occurs, H_2S and sodium salt of L-arginine is produced which are responsible for individual pharmacological responses, which further needs to be verified in *in vivo* studies.

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CLINDAMYCIN: EFFECTS ON PLASMA LIPID PROFILE AND PEROXIDATION PARAMETERS IN RABBIT BLOOD PLASMA

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Abstract: Alteration of plasma lipid profile and induction of lipid peroxidation may take place due to drug effect, which may be correlated with adverse drug reactions and drug-induced toxicity. Considering this fact, the present *in vivo* study was carried out to evaluate the effect of clindamycin on plasma lipid profile and peroxidation parameters alone and in combination with ascorbic acid, a promising antioxidant. After administering drug and antioxidant alone and in combination in rabbit, it was found that clindamycin had mild lipid peroxidation induction and profile alteration capacity, which can be arrested on co-administration of ascorbic acid.

Keywords: clindamycin, adverse drug reactions, lipid peroxidation, lipid profile, ascorbic acid

During the passage of transport, drug has to cross many barriers, most of which are lipoidal in nature. Interaction of drug with membrane lipids may lead to alteration in lipid pattern and composition, as well as lipid peroxidation, which is a measure of the membrane damage. Lipid peroxidation is the oxidative deterioration of polyunsaturated lipids (1). It is a highly destructive process that induces a wide variety of alterations in the structure and function of cellular membranes (2), and an important cause of certain diseases or disorders, like diabetes mellitus, gastric ulcer, neurodegenerative diseases (3), atherosclerosis (4), aging (5) etc. Indomethacin-induced gastric mucosal damage (6) and doxorubicin-induced cardiomyopathy (7) are the consequences of drug-induced lipid peroxidation and reactive oxygen species (ROS) formation.

A drug may also induce changes in plasma lipid profile (8-11). A lipid profile is a measure of three components: cholesterol (Ch), triglycerides (Tg), and lipoproteins (LP) (high and low density). Total cholesterol (TCh) comprises all the cholesterol found in various lipoproteins, such as high density LP (HDL), low density LP (LDL) and very low density LP (VLDL). High density lipoprotein cholesterol (HDL-Ch) is believed to play a key role in the process of reverse cholesterol transport that promotes the efflux of excess cholesterol from vessel wall to the liver for excretion (12, 13). On the contrary, low density lipoprotein cholesterol (LDL-Ch) contains the high percentage of cholesterol, and is responsible for cholesterol deposit on the arterial wall, resulting in coronary symptom like atherosclerosis. Very low density lipoprotein cholesterol (VLDL-Ch) is a large group of macromolecules synthesized and secreted mainly by liver and intestinal mucosal cells, and contains large quantities of Tg (14). Tg is the neutral fat metabolite found in the tissue and blood and may contribute to the disorders related to coronary heart disease (CHD) (15). Phospholipid (PL) is one of the major components of total lipid (TL) present in the biological membrane. Evidence suggests that oxidized PL (a major lipid of LDL-Ch) is formed in atherogenesis and plays an important role in the oxidative modification during LDL-Ch oxidation (16). Elevated lipid profile is associated with many diseases. Diabetic patients have increased level of TCh, Tg, LDL-Ch, VLDL-Ch and decreased level of HDL-Ch (17). CHD is also associated with elevated level of blood TCh, Tg, LDL-Ch and decreased HDL-Ch (18). Blood TCh, HDL-Ch and LDL-Ch are found to be

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inversely associated with incidence of cancer like leukemia and Hodgkin's disease, but Tg is found to be significantly elevated in patients (19). In AIDS patients, disease progression is accompanied by a decrease in TCh, HDL-Ch and LDL-Ch, and increase in Tg and VLDL-Ch levels (20). Patients with chronic kidney disease (CKD) are at an increased risk for cardiovascular disease and have a higher prevalence of hyperlipidemia (21).

Clindamycin is a lincosamide antibiotic similar in mechanism of action and spectrum of activity to erythromycin (22). It inhibits most gram positive cocci, *C. diphtheriae*, *Nocardia*, *Actinomyces*, *Toxoplasma*, but the distinctive feature is its high activity against a variety of anaerobes specially *Bact. fragilis*. But the use of this antibiotic is restricted due to the development of adverse reactions including pseudomembranous enterocolitis which is potentially fatal (22). In the present *in vivo* study, an attempt has been made to evaluate the lipid peroxidation induction and lipid profile alteration potential of clindamycin and their subsequent control on ascorbic acid co-administration. Ascorbic acid, a promising antioxidant, has free radical scavenging capacity (23, 24). Use of antioxidants as adjuvant therapy may become a promising approach (8, 9, 25) in reducing drug-induced abnormalities. Alteration of lipid profile, which may occur due to drug effect, is also regulated by antioxidant ascorbic acid.

EXPERIMENTAL

Lipid peroxidation induction potential of the drug was measured by estimating laboratory markers, like malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), reduced glutathione (GSH) and nitric oxide (NO) levels, and lipid profile alteration ability was measured by estimating TCh, HDL-Ch, Tg, LDL-Ch, VLDL-Ch, PL and TL levels. New Zealand white rabbits (*Oryctolagus cuniculus*) were used as animal model. All the reagents used in the study were of analytical grade. The design of the study protocol was approved by Institutional Animal Ethical Committee.

Collection of blood

Animals were divided into different experimental groups: control (C), drug treated (D), drug co-administered with antioxidant (DA) and only antioxidant treated (A). The drug, clindamycin was administered intramuscularly at a dose of 30 mg/kg body weight (26) to animal groups marked as D and DA. The antioxidant, ascorbic acid was administered at a dose of 40 mg/kg body weight (27) to animal groups marked as DA and A. Blood was collected from marginal ear vein of animal after 3 and 24 h of drug and/or antioxidant administration and the samples were subjected to test for determination of effect of drug and antioxidant on peroxidation parameters and lipid profiles.

Determination of lipid peroxidation

Drug-induced lipid peroxidation was measured by estimating the content of MDA, 4-HNE, GSH and NO in blood sample. Determination was done by precipitating the protein substances using trichloroacetic acid (10% w/v). The protein free samples were used for estimation of lipid peroxidation parameters as follows:

Estimation of MDA

The protein free sample was added to equal volume of thiobarbituric acid (TBA) and heated in a boiling water bath for 30 min. The absorbance of the pink colored sample was measured at 530 nm against a blank (28). The concentration of MDA present in the sample was estimated from the standard curve prepared using tetraethoxypropane (TEP) and TBA (1 : 1).

Estimation of 4-HNE

The sample was mixed (1 : 1) with 2,4-dinitrophenylhydrazine (DNPH) solution (100 mg% in 0.5 M HCl) and incubated at room temperature for 1 h. The mixture was extracted with hexane followed by addition of methanol. The absorbance of the methanol sample was measured at 350 nm (29). The concentration was estimated from the standard curve.

Estimation of GSH

GSH was measured by reacting the sample with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) to give a color complex (Ellman's method) (30). The protein free sample was mixed with DTNB (1 : 3) solution (0.01% in phosphate buffer 0.1 M, pH 8) and absorbance of the solution was measured at 412 nm against a blank. Concentration of GSH present in the blood samples was estimated from the standard curve.

Estimation of NO

NO content was determined by reaction with Griess reagent [1 : 1 sulfanilamide (1% w/v in 3 M HCl) and 0.1% w/v N-(1-naphthyl)ethylenediamine dihydrochloride]. The pH of the mixture was adjusted to 6.7 with Na_2HPO_4 and the absorbance of the

solutions was measured at 540 nm (31). The concentration of NO was estimated from the standard curve.

The percent changes in peroxidation parameters, MDA, GSH, 4-HNE, and NO levels of different samples at different hours of interval were calculated with respect to the control.

Determination of lipid profiles

Drug-induced changes in lipid profile were measured by estimating the level of TCh, HDL-Ch, LDL-Ch, VLDL-Ch, Tg, PL and TL in the blood serum. The commercially available enzyme kits used for estimation of lipid profiles were obtained from Span Diagnostics Ltd., Surat, India and Labkit, Barcelona, Spain.

Estimation of TCh

The total cholesterol was estimated by cholesterol oxidase (CHOD) – peroxidase aminoantipyrine phenol (PAP) method (32, 33). Ten microliters of blood serum was mixed with 1 mL of cholesterol reagent, containing Good's buffer pH 6.7, cholesterol esterase, cholesterol oxidase, peroxidase, 4-aminoantipyrine and stabilizers. The mixture was incubated at 37^{o}C for 10 min. The absorbance was measured at 505 nm against cholesterol reagent as blank. The concentration of TCh was calculated from the standard curve prepared using cholesterol standard samples.

Estimation of HDL-Ch

Estimation of HDL cholesterol was done using CHOD – PAP method (33). Two hundred microliters of serum was mixed with 200 µL of precipitating reagent containing PEG 6000 (200 mM/L), stabilizer and preservative. The mixture was kept at room temperature for 10 min and centrifuged for 15 min at 2000 rpm and the clear supernatant was separated. 100 µL of supernatant was mixed with 1 mL

Table 1. Percent changes in lipid peroxidation parameters with respect to control.

Parameter	Average ((± SE) % change at time interval							
	3 h				**24 h**			
	D	DA	A	ANOVA and multiple comparison	D	DA	A	ANOVA and multiple comparison
MDA	8.19 (± 0.71)	4.02 (± 0.29)	-8.60 (± 0.62)	F1 = 295.53 (df 2,8) F2 = 1.83 (df 4,8) Pooled variance = 1.29 LSD = 1.56 Ranked means$^{\$}$ = (D) (DA) (A)	2.01 (± 0.29)	0.55 (± 0.24)	-2.34 (± 0.17)	F1 = 98.20 (df 2,8) F2 = 1.49 (df 4,8) Pooled variance = 0.25 LSD = 0.69 Ranked means$^{\$}$ = (D) (DA) (A)
4-HNE	7.14 (± 1.02)	3.16 (± 0.64)	-8.79 (± 0.29)	F1 = 177.69 (df 2,8) F2 = 2.04 (df 4,8) Pooled variance = 1.93 LSD = 1.91 Ranked means$^{\$}$ = (D) (DA) (A)	2.23 (± 0.51)	0.87 (± 0.14)	-2.49 (± 0.16)	F1 = 64.44 (df 2,8) F2 = 1.42 (df 4,8) Pooled variance = 0.46 LSD = 0.93 Ranked means$^{\$}$ = (D) (DA) (A)
GSH	-7.56 (± 0.67)	-3.77 (± 0.42)	4.46 (± 0.78)	F1 = 85.49 (df 2,8) F2 = 0.84 (df 4,8) Pooled variance = 2.20 LSD = 2.04 Ranked means$^{\$}$ = (D) (DA) (A)	-2.05 (± 0.15)	0.17* (± 0.94)	1.27 (± 0.31)	F1 = 9.02 (df 2,8) F2 = 1.19 (df 4,8) Pooled variance = 1.58 LSD = 1.73 Ranked means$^{\$}$ = (D) (DA, A)
NO	-10.09 (± 1.13)	-5.43 (± 0.61)	17.72 (± 2.51)	F1 = 86.06 (df 2,8) F2 = 1.11 (df 4,8) Pooled variance = 12.88 LSD = 4.94 Ranked means$^{\$}$ = (D, DA) (A)	-4.84 (± 0.65)	-4.01 (± 0.87)	6.06 (± 0.86)	F1 = 74.05 (df 2,8) F2 = 1.91 (df 4,8) Pooled variance = 2.48 LSD = 2.17 Ranked means$^{\$}$ = (D, DA) (A)

Average (of 5 animal sets) percent changes with respect to control of corresponding hours are shown. Reproducibility measured by 't' test and the values are significant at $p < 0.05$ except marked with* F1 and F2 correspond to variance ratio between samples and between animals, respectively. LSD means critical difference according to the least significant difference procedure. D, DA and A indicate clindamycin-treated, clindamycin and ascorbic acid-treated and only ascorbic acid-treated, respectively. SE = standard error (df = 4); df = degrees of freedom. $^{\$}$ denotes that two means not included within the same parenthesis are statistically significantly different at $p < 0.05$

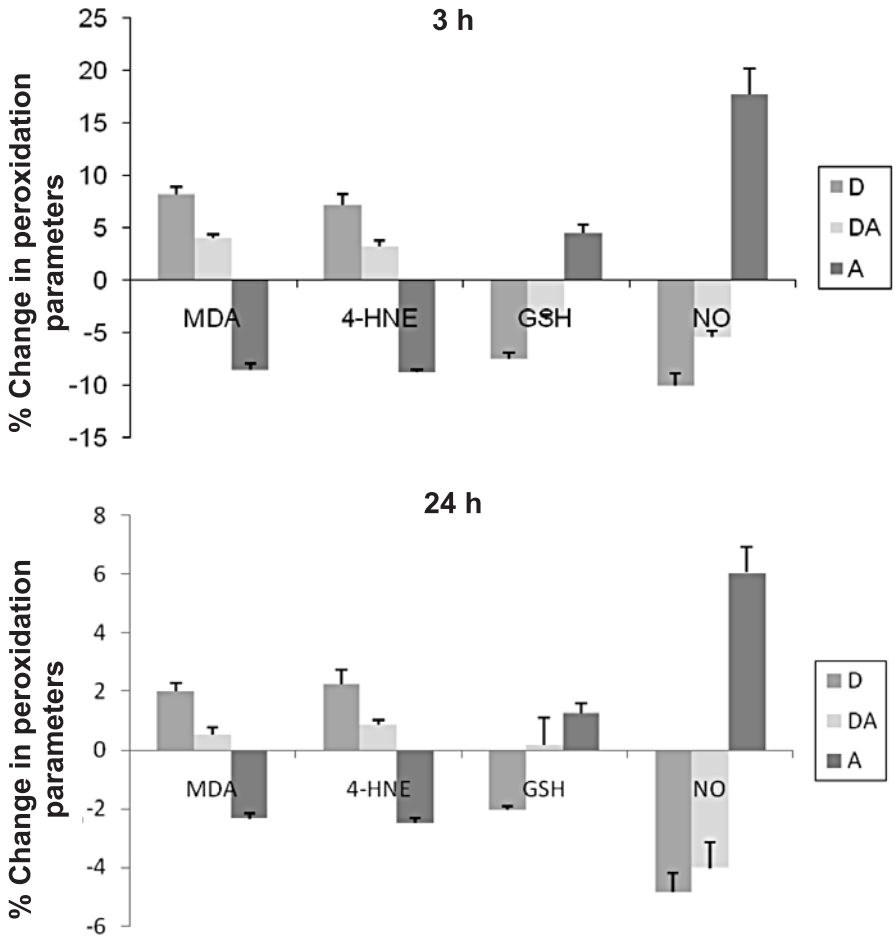

Figure 1. Effect of clindamycin and ascorbic acid on lipid peroxidation parameters. D, DA and A denote clindamycin treated, clindamycin-ascorbic acid treated and only ascorbic acid treated animals, respectively, MDA = malondialdehyde, 4-HNE = 4-hydroxy-2-nonenal, GSH = reduced glutathione, NO = nitric oxide

of cholesterol reagent and incubated at 37°C for 10 min. The absorbance was measured at 505 nm. The concentration of HDL-Ch was calculated from a standard curve prepared using HDL-Ch standard samples.

Estimation of LDL-Ch and VLDL-Ch

Friedewald's equations (34) were used to calculate concentrations of LDL-Ch and VLDL-Ch in the samples.

LDL-Ch content = TCh content – (Tg content / 5) – HDL-Ch content

VLDL-Ch content = Tg content / 5

Estimation of Tg

Ten microliters of serum was mixed with 1 mL of Tg mono reagent containing pipes buffer, 4-chlorophenol, magnesium, ATP, lipase, peroxidase, glycerol kinase, 4-aminoantipyrine, glycerol-3-phosphate oxidase, detergents, preservative and stabilizer. The mixture was incubated at 37°C for 10 min. The absorbance of the solution was measured at a wavelength of 505 nm (33, 35). The concentration of Tg was calculated from a standard curve prepared using Tg standard samples.

Estimation of PL

Ten microliters of blood serum was mixed with 1 mL of reagent containing TRIS buffer pH 7.55, dichlorophenol, phospholipase D, choline oxidase, peroxidase and 4-aminophenazone. The mixture was incubated for 5 min at 37°C and the absorbance of the solution was measured at a wavelength of 505 nm (36). The concentration of PL was calculated from a standard curve prepared using PL primary standards.

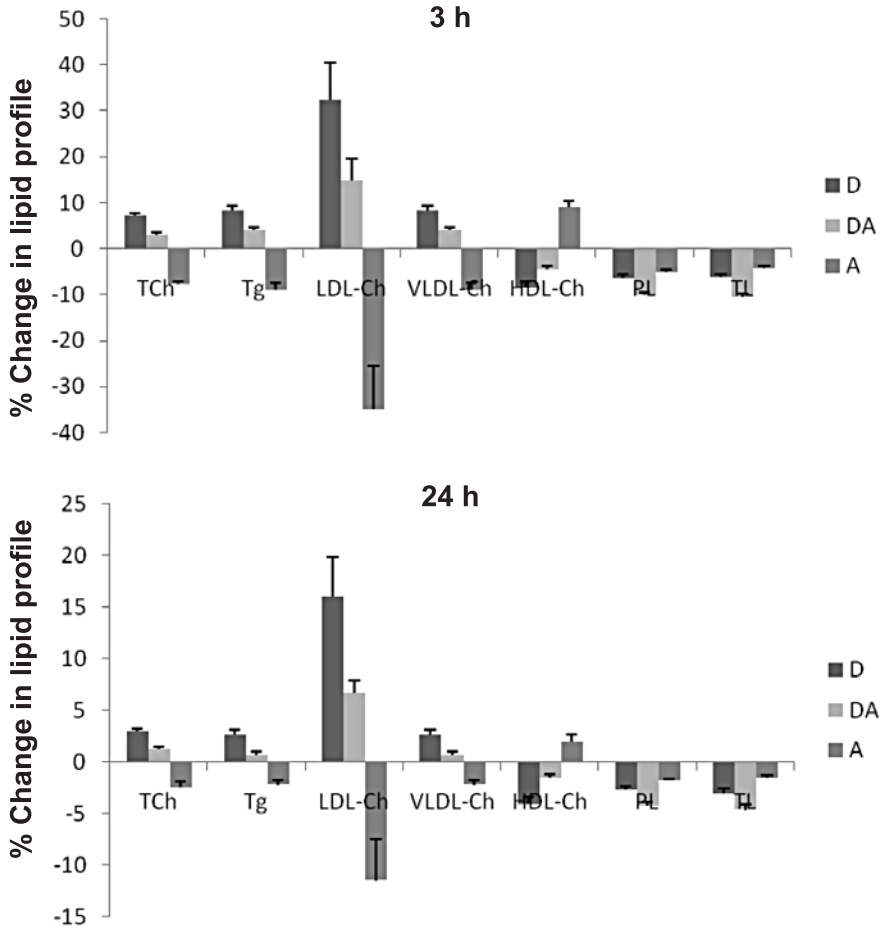

Figure 2. Effect of clindamycin and ascorbic acid on lipid profile. D, DA and A denote clindamycin treated, clindamycin-ascorbic acid treated and only ascorbic acid treated animals, respectively. TCh = total cholesterol, Tg = triglyceride, LDL-Ch = LDL cholesterol, VLDL-Ch = VLDL cholesterol, HDL-Ch = HDL cholesterol, PL = phospholipid, TL = total lipid

Estimation of TL

Hundred microliters of serum was mixed with 2.5 mL of sulfuric acid, heated for 10 min in a boiling water bath (100^{o}C) and then cooled in iced water. Fifty microliters of the acid digested sample was mixed with 1 mL of phosphovanillin reagent and incubated for 15 min at 37^{o}C. The absorbance was measured at a wavelength of 520 nm (37, 38). The concentration of TL present in the sample was calculated from a standard curve prepared using TL primary standards.

The percent changes in TCh, HDL-Ch, LDL-Ch, VLDL-Ch Tg, PL and TL levels of different samples at different time intervals were calculated with respect to the control.

RESULTS AND DISCUSSION

Results of the study are presented in Tables 1, 2 and are further illustrated in Figures 1, 2. The results were statistically validated by analysis of variance (ANOVA) followed by multiple comparison using a least significant difference procedure (39, 40). From Table 1 and Figure 1, it is evident that clindamycin has low but significant lipid peroxidation induction potential that might cause elevation of MDA and 4-HNE contents, which were found to be the end products of lipid peroxidation (41) and involved in drug-induced toxicity (6, 7). When ascorbic acid was co-administered, it reduced the elevated levels of MDA and 4-HNE. Results (Table 1 and Figure 1) also showed the reduction in GSH and NO contents that might be due to peroxidation induction capacity of clindamycin. GSH and NO are related to the antioxidant defense mechanism (42, 43) of the body. Again, when animals received both drug and antioxidant, the GSH and NO levels are elevated with respect to the drug treated group. Levels of these parameters are increased compared to the control group in animals which

received only ascorbic acid. This increased GSH and NO contents indicate the antiperoxidative potential of ascorbic acid (8, 9, 25).

Drug-induced alteration in lipid profile and their control by ascorbic acid are illustrated in Table 2 and Figure 2. From the results, it is evident that clindamycin slightly elevated the levels of TCh, LDL-Ch, VLDL-Ch and Tg, which are further decreased upon co-administration of ascorbic acid. Both Table 2 and Figure 2 showed a reduced level of

Table 2. Percent changes in lipid profile contents with respect to control.

Parameter	Average ((± SE) % change at time interval							
	3 h				**24 h**			
	D	DA	A	ANOVA and multiple comparison	D	DA	A	ANOVA and multiple comparison
TCh	7.19 (± 0.63)	3.00 (± 0.49)	-7.78 (± 0.67)	F1 = 125.46 (df 2,8) F2 = 0.31 (df 4,8) Pooled varianc e= 2.37 LSD = 2.12 Ranked means$ = (D) (DA) (A)	2.93 (± 0.25)	1.21 (± 0.25)	-2.56 (± 0.60)	F1 = 37.67 (df 2,8) F2 = 0.34 (df 4,8) Pooled variance = 1.04 LSD = 1.40 Ranked means$ = (D) (DA) (A)
Tg	8.23 (± 1.09)	4.04 (± 0.45)	-9.11 (± 1.62)	F1 = 46.29 (df 2,8) F2 = 0.28 (df 4,8) Pooled variance = 8.84 LSD = 4.09 Ranked means$ = (D) (DA) (A)	2.56 (± 0.49)	0.62 (± 0.35)	-2.23 (± 0.41)	F1 = 24.81 (df 2,8) F2 = 0.32 (df 4,8) Pooled variance = 1.17 LSD = 1.49 Ranked means$ = (D) (DA) (A)
LDL-Ch	32.48 (± 7.90)	14.75 (± 4.91)	-34.92 (± 9.36)	F1 = 15.75 (df 2,8) F2 = 0.25 (df 4,8) Pooled variance = 387.33 LSD = 27.12 Ranked means$ = (D, DA) (A)	16.00 (± 3.84)	6.69 (± 1.14)	-11.55 (± 4.01)	F1 = 13.06 (df 2,8) F2 = 0.14 (df 4,8) Pooled variance = 75.21 LSD = 11.95 Ranked means$ = (D, DA) (A)
VLDL-Ch	8.23 (± 1.09)	4.04 (± 0.45)	-9.11 (± 1.62)	F1 = 46.29 (df 2,8) F2 = 0.28 (df 4,8) Pooled variance = 8.84 LSD = 4.09 Ranked means$ = (D) (DA) (A)	2.56 (± 0.49)	0.62 (± 0.35)	-2.23 (± 0.41)	F1 = 24.81 (df 2,8) F2 = 0.32 (df 4,8) Pooled variance = 1.17 LSD = 1.49 Ranked means$ = (D) (DA) (A)
HDL-Ch	-8.45 (± 1.32)	-4.48 (± 0.71)	9.01 (± 1.28)	F1 = 46.51 (df 2,8) F2 = 0.17 (df 4,8) Pooled variance = 8.99 LSD = 4.13 Ranked means$ = (D, DA) (A)	-4.18 (± 0.71)	-1.57 (± 0.34)	1.95 (± 0.66)	F1 = 18.56 (df 2,8) F2 = 0.11 (df 4,8) Pooled variance = 2.54 LSD = 2.19 Ranked means$ = (D) (DA) (A)
PL	-6.30 (± 0.76)	-9.99 (± 0.54)	-5.05 (± 0.50)	F1 = 33.05 (df 2,8) F2 = 3.68 (df 4,8) Pooled variance = 0.99 LSD = 1.37 Ranked means$ = (D, A) (DA)	-2.74 (± 0.34)	-4.28 (± 0.43)	-1.84 (± 0.17)	F1 = 41.26 (df 2,8) F2 = 7.20 (df 4,8) Pooled variance = 0.18 LSD = 0.58 Ranked means$ = (D) (DA) (A)
TL	-6.03 (± 0.49)	-10.50 (± 0.79)	-4.20 (± 0.45)	F1 = 94.67 (df 2,8) F2 = 7.73 (df 4,8) Pooled variance = 0.55 LSD = 1.02 Ranked means$ = (D) (DA) (A)	-3.07 (± 0.49)	-4.67 (± 0.58)	-1.60 (± 0.21)	F1 = 24.70 (df 2,8) F2 = 4.61 (df 4,8) Pooled variance = 0.48 LSD = 0.95 Ranked means$ = (D) (DA) (A)

Average (of 5 animal sets) percent changes with respect to control of corresponding hours are shown. Reproducibility measured by 't' test and the values are significant at $p < 0.05$. F1 and F2 correspond to variance ratio between samples and between animals, respectively. LSD means critical difference according to the least significant difference procedure. D, DA and A indicate clindamycin-treated, clindamycin and ascorbic acid-treated and only ascorbic acid-treated, respectively. SE = standard error (df = 4); df = degrees of freedom. $ denotes that two means not included within the same parenthesis are statistically significantly different at $p < 0.05$

HDL-Ch in drug treated animals with respect to control. The group receiving both drug and antioxidant showed increased level of HDL-Ch than the drug treated group, while only antioxidant treated group showed elevated level of HDL-Ch in comparison to control animals. Elevation in TCh, LDL-Ch, VLDL-Ch, Tg contents and reduction in HDL-Ch may be linked with drug-induced alteration in lipid profile (8, 9, 44-46), though changes in lipoprotein level may also happen as a result of drug-induced protein metabolism (47). The results further corroborated with lipid peroxidation induction potential of the drug that caused elevation of TCh, Tg, LDL-Ch and VLDL-Ch and reduction in HDL-Ch levels (8, 9). The present study also showed that the liposuppressive effect of the antioxidant ascorbic acid plays a beneficial role to minimize abnormal lipid profile alteration including increased lipid peroxidation (48-51). Ascorbic acid is not only an antioxidant but also an important antihyperlipidemic agent. From Table 2 and Figure 2 it was also found that there is a reduction in PL and TL contents in all animal groups. In case of drug treated group, the reduction in PL and TL contents might be due to binding ability of the drug with lipids (52). The efficient binding capacity of the drugs to PL and TL might cause significant reduction of those parameters, and increased level of peroxidation parameter like MDA (53, 54).

In both figures, similar pattern of changes occurs in both 3 and 24 h time period, but the extent of change is more prominent in the former. This difference may be due to significant elimination of the drug and antioxidant from the body within 24 h.

The drug – clindamycin – is found to be effective in the treatment of many bacterial infections (22) and has also mild lipid peroxidation induction potential. The drug-induced changes in lipid profile may be correlated with its lipid peroxidation induction potential. Some of the toxicities of clindamycin may be linked with its lipid peroxidation induction capacity and that can be effectively controlled on co-administration of ascorbic acid, having free radical scavenging capacity (23, 24). The concept of antioxidant co-therapy may be exploited during future formulation design with an aim of reducing drug-induced adverse reactions and toxicities. Moreover, lipid peroxidation induction as well as lipid profile alteration capacity of a drug may be tested at the individual level to determine the extent of risk from the drug in case of a particular individual in view of variable *in vivo* antioxidant defense and accordingly, the decision about safe use of a drug and necessary co-administration of antioxidant may be taken. Thus, the antioxidant co-therapy approach will be an effective tool for the physicians to reduce possibilities of drug-related hazards. This may lead to enhancement of patient compliance and improvement of therapeutic index of the drug.

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EVALUATION OF SELECTED BIOCHEMICAL PARAMETERS OF OXIDATIVE STRESS IN RATS PRETREATED WITH ROSUVASTATIN AND FLUOXETINE

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Abstract: The aim of this study was to assess the effect of a combined 14-day treatment with rosuvastatin (10 mg/kg) and fluoxetine (10 mg/kg) on selected biochemical parameters of oxidative stress in the blood of rats. The activity of glutathione peroxidase (GPX), glutathione reductase (GR) and the total antioxidant status (TAS) were determined. A combined 14-day treatment with rosuvastatin and fluoxetine significantly increases glutathione peroxidase and glutathione reductase activity and decreases the level of TAS. Rosuvastatin administered to rats caused a decrease in the glutathione peroxidase activity and an increase in the glutathione reductase activity but did not affect the level of TAS. A 14-day treatment with fluoxetine exerted practically no effect on the investigated parameters of oxidative stress in rats. The abovementioned changes in the biochemical parameters after the combined treatment with rosuvastatin and fluoxetine may imply an imbalance in prooxidant and antioxidant levels in the combined treatment with rosuvastatin and fluoxetine.

Keywords: rosuvastatin, fluoxetine, rats, oxidative stress parameters

Cardiovascular diseases, such as hypertension, coronary heart disease, heart failure and stroke, are the leading cause of mortality worldwide and their incidence rises. Reactive oxygen species (ROS) and reduced antioxidant enzymatic defense are important factors in the pathogenesis of cardiovascular disorders. Oxidative stress is defined as a disturbance in the balance between the production of ROS and antioxidant defense (1). ROS are highly reactive and may oxidize and damage important components such as proteins or DNA. They cause changes in biological molecules; these changes accumulate over time in the biological structures, which may lead to the molecular damage of tissue structure (2). Statins (3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors) are widely used in the clinical practice for reducing morbidity and mortality attributed to cardiovascular diseases. Experimental and clinical studies have shown that statins may also have important antiinflammatory effects through their ability to block the production and activity of reactive oxygen species (3). Rosuvastatin is a potent statin displaying pharmacologic and pharmacokinetic advantages. It has a superior efficacy in lowering LDL-C as well as improving HDL-C. Rosuvastatin cholesterol-independent effects include the modulation of vascular and inflammatory response and antioxidant activity (4-6). Rosuvastatin maintains the balance between oxidant generation and oxidant scavenging (7). Our previous studies conducted on rats also indicated a positive effect of rosuvastatin in this regard (8).

Depressive disorders are common for patients with cardiovascular diseases. Several studies have shown that depression and its associated symptoms are a major risk factor for both the development of cardiovascular disease and death after an index myocardial infarction (9). Therefore, the treatment of a depressed patient with a cardiovascular disease is necessary. The selective serotonin reuptake inhibitors (SSRIs) appear to be a relatively safe and effective treatment for depression in patients with a comorbid heart disease (10). Fluoxetine is a commonly chosen first-line medication because it is not associated with the cardiovascular effects (11). A combined treatment with rosuvastatin and fluoxetine may lead to the weakening of the antioxidant properties of rosuvastatin, which may be crucial in a therapy with this drug. Moreover, a long-term combined therapy can also cause oxidation-reduction imbalance and an increase in the generation of ROS that can cause oxidative stress.

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The purpose of the study was to evaluate some selected biochemical parameters of oxidative stress in the blood of rats pretreated with rosuvastatin and fluoxetine. After 14 days of intraperitoneal (*i.p.*) application of the drug, alone or in combination, the activity of glutathione peroxidase (GPX), glutathione reductase (GR) and also total antioxidant status (TAS) were determined. GPX appears in many tissues, first of all in the liver and blood. Its main role is to protect cells from oxidative stress, especially from hydrogen peroxide. GPX is closely connected with glutathione reductase (12). The determination of the total antioxidant status can serve for the establishment of the antioxidant potential of the drug and can indicate whether the treatment has any adverse effect on the antioxidation system.

MATERIALS AND METHODS

Animals

The study was carried out on male Wistar rats weighing initially 200-250 g and obtained from a licensed breeder. The animals were kept in room temperature (20 ± 1°C) under a natural day-night cycle in constant environmental conditions. The rats had access to food and water *ad libitum*. The study was approved by the Ethical Committee for Animal Experimentation of the Medical University of Lublin.

Drugs and chemicals

The following drugs were used in our study: rosuvastatin (Romazic tabl., Polpharma SA, Poland), fluoxetine (Fluoksetyna, Anpharm, Poland), *aqua pro injectione* (Baxter, Poland). Ready-made diagnostic kits (RANDOX Laboratories Ltd., Antrim, U. K.) were used to determine: GPX, GR and TAS.

Experimental protocols

Aqueous solutions of rosuvastatin (10 mg/kg) and fluoxetine (10 mg/kg) were prepared *ex tempore* and administered *i.p.* once daily for 14 days alone or in combination in the constant volume of 0.5 mL/100 g of body weight. The control groups were given the appropriate amounts of *aqua pro injectione*. The experimental groups consisted of eight animals each. Twenty four hours after the last injection, the animals were decapitated, and the blood was taken. One part of the blood was collected in heparin tubes (whole blood) and the other as clot. The whole heparinized blood was used to estimate the GP activity. The other part of the blood, as indicated above, was left to clot. The serum fraction was separated and taken in order to determine the GR activity and TAS.

Statistical analysis

All statistical calculations were carried out with the ANOVA test, and p-values less than 0.05

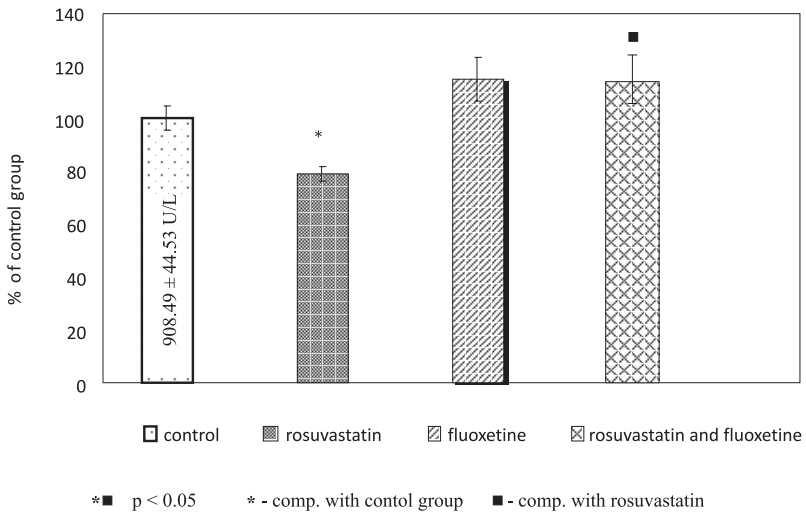

Figure 1. Effect of 14-day treatment with rosuvastatin (10 mg/kg) and fluoxetine (10 mg/kg) on glutathione peroxidase activity

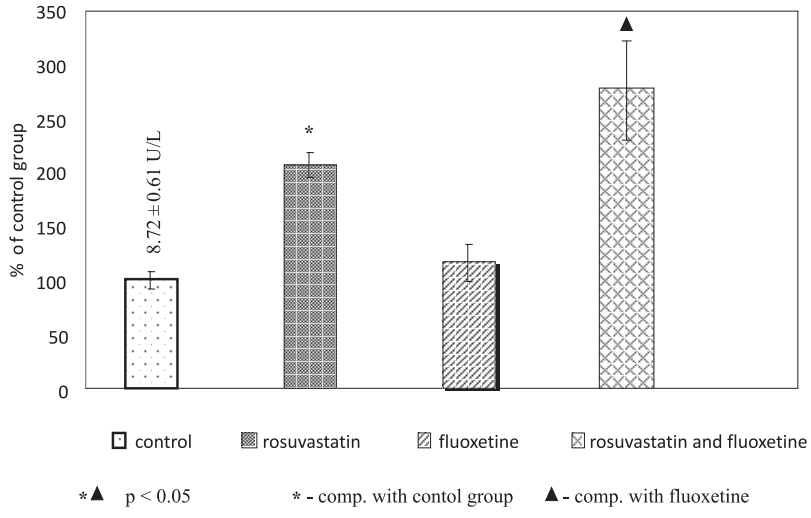

Figure 2. Effect of 14-day treatment with rosuvastatin (10 mg/kg) and fluoxetine (10 mg/kg) on glutathione reductase activity

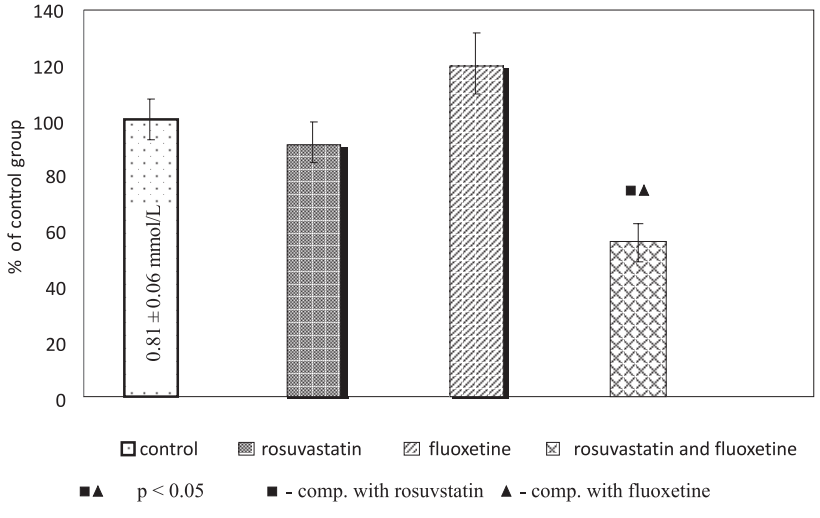

Figure 3. Effect of 14-day treatment with rosuvastatin (10 mg/kg) and fluoxetine (10 mg/kg) on total antioxidant status level

were considered significant. The results were expressed as the mean ± SEM.

RESULTS

We observed that rosuvastatin (10 mg/kg) administered to rats for 14 days causes a decrease of the GP activity compared with the control group (Fig. 1). Fluoxetine (10 mg/kg) administered to rats did not significant affect the activity of GP. However, rosuvastatin administered for 14 day simultaneously with fluoxetine causes an increase of the GP activity in rat blood compared with the group of animals receiving only rosuvastatin.

A two-week administration of rosuvastatin to rats increases the activity of GR compared with the control group (Fig. 2). In the group of rats receiving only fluoxetine, any significant changes in the activity of GR were recorded and compared with the control group. The combined treatment of rats with

rosuvastatin and fluoxetine causes a significant increase of the activity of GR when compared with the groups of animals receiving only fluoxetine.

The group of rats receiving only rosuvastatin or fluoxetine did not demonstrate any significant changes of the level of TAS when compared with the control group (Fig. 3), whereas the combined treatment with rosuvastatin and fluoxetine results in the decrease of TAS in the serum of rats compared with the groups treated with these drugs alone.

DISCUSSION

Hyperlipidemia and elevated plasma low-density lipoprotein are considered relevant risk factors for the emergence of cardiovascular diseases, the major cause of mortality in Western populations. Also the intensification of the oxidative stress and decreased antioxidant capacity are likely to contribute to the increased risk of a cardiovascular disease (2).

As shown in clinical studies, a rosuvastatin therapy not only leads to a reduction of cholesterol but also significantly reduces oxidative stress and has further beneficial immunomodulatory and thus antiinflammatory effects, which may lead to the reduction of risk of atherosclerosis and cardiovascular diseases (4, 6, 13).

We observed that a two-week treatment with rosuvastatin reduces the activity of GP, increases the activity of GR, yet has no influence on TAS. Similar results were obtained in our previous study (8). GP is an enzyme present mainly in blood and liver, and its main task is to protect cells against oxidative stress, especially against hydrogen peroxide. This enzyme catalyzes the reaction of hydrogen peroxide and organic peroxides by reduced glutathione (14). The final product of the reaction is glutathione disulfide (GSSG). GSSG is harmful to cells because it oxidizes the thiol groups of proteins and leads to their inactivation. GP remains closely connected with GR which reproduces a reduced form of glutathione. A decrease in the activity of GP may confirm the beneficial effect of rosuvastatin consisting in restraining the formation of ROS. Also an increased activity of GR may suggest the protective effect of rosuvastatin aimed at maintaining an adequate level of the reduced form of glutathione and preventing the accumulation of hydrogen peroxide.

Some studies suggested a beneficial effect of fluoxetine in reducing oxidative stress (15, 16). In our research, fluoxetine administered to rats has no significant effect on the determined parameters. However, a 14-day combined treatment with rosuvastatin and fluoxetine yields significant changes in the assayed biochemical parameters. A combined treatment with these drugs significantly enhances the activity of GP in comparison with the group of rats receiving rosuvastatin, which may indicate a diminished antioxidant activity of rosuvastatin. A simultaneous application of rosuvastatin and fluoxetine causes an increased activity of GR compared with the group of rats receiving only fluoxetine, while causing no significant change compared with the group treated with rosuvastatin. A significant increase in the activity of GR in relation to the control group may indicate an increased production of hydrogen peroxide. Two weeks of a simultaneous treatment with rosuvastatin and fluoxetine proved a decrease of the total antioxidant status in comparison with the groups of rats receiving both drugs separately. The total antioxidant status, defined as an ability of the serum to quench free radical production, consists in a multicompartmental protection against molecular damage of the cell structure. TAS is sensitive to changes in the plasma antioxidant levels and degrees of oxidative stress (2). A decrease in the level of TAS suggests an increase in the generation of oxygen free radicals and a decrease in the antioxidant defense system (17, 18). Thus, the observations after the combined drug treatment covered by this study suggest increased oxidant stress and decreased antioxidant levels. In turn, our previous studies carried out on rats treated simultaneously with rosuvastatin and amitriptyline (tricyclic antidepressant (TCA)) indicated an increased total antioxidant status (8). These results are surprising because SSRIs seem to be relatively safer than TCAs. Perhaps, these differences result from the shared metabolism of rosuvastatin and fluoxetine. These drugs are biotransformed by cytochrome P450 izoenzyme CYP2C9, while this izoenzyme is not involved in the metabolism of amitriptyline.

CONCLUSIONS

Rosuvastatin (10 mg/kg) administered to rats for 14 days causes a decrease in the GP activity and an increase in the GR activity but does not affect the level of TAS.

A 14-day treatment with fluoxetine (10 mg/kg) has practically no effect on the investigated parameters of oxidative stress in rats.

A 14-day combined treatment with rosuvastatin and fluoxetine causes a significant increase in the glutathione peroxidase and glutathione reductase activity but reduces the level of TAS.

The changes observed in the examined parameters may suggest an imbalance in the prooxidant and antioxidant levels in the combined treatment with rosuvastatin and fluoxetine.

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INFLUENCE OF CLOUDY APPLE JUICE ON N-NITROSODIETHYLAMINE-INDUCED LIVER INJURY AND PHASES I AND II BIOTRANSFORMATION ENZYMES IN RAT LIVER

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Abstract: Cloudy apple juice (CAJ) is a rich source of nutrients as well as non-nutrient components including high quantity of polyphenols, particularly oligomeric procyanidins, which are considered as potential chemo-preventive agents that protect against the action of chemical carcinogens. The aim of this study was to examine the effect of CAJ alone or in combination with hepatocarcinogenic N-nitrosodiethylamine (NDEA) on liver damage biomarkers, including DNA damage, and the phase I and II enzymes in rat. The forced feeding with CAJ alone for 28 days, has slightly reduced the activities of phase I enzymes, MROD (CYP1A2 biomarker) and PNPH (CYP2E1 biomarker), while phase II enzymes, glutathione S-transferase (GST) and NAD(P)H:quinone oxidoreductase-1 (NQO1), were elevated. Combined treatment of rats with CAJ and NDEA significantly reduced the levels of hepatic ALT and SDH (by ~100%) as compared to values from NDEA-treated animals. CAJ pretreatment further increased the PROD (CYP2B biomarker) and NQO1 activities increased by NDEA administration. Modulation of enzymes activities was accompanied by the changes in the proteins levels. These results indicate that CAJ may protect liver against damage induced by NDEA. Moreover, a significant decrease of SDH activity by CAJ may confirm its potential anti-diabetic activity.

Keywords: cloudy apple juice, cytochrome P450, GST, NDEA, NQO1, comet assay

Apples and apple juice are the most widely consumed fruit and fruit products in the Western diet. Early epidemiological studies have linked the consumption of apples with reduced risk of certain cancers, cardiovascular disease, asthma and diabetes (1). Experimental data indicated the antioxidant, anti-inflammatory and anti-mutagenic activities of apples or apple juice, which might be related to signal transduction pathways and carcinogen metabolism modulation (1, 2).

Apples are a rich source of phenolic constituents, which are distributed in the peel, core and pulp. The content and composition of phenolic compounds vary strongly depending on the apple variety, area of cultivation and time and year of harvest. The total polyphenol content of apples represents about 0.01 to 1% of the fresh weight. The main structural classes include hydroxycinnamic acids, dihydrochalcones, flavonols (quercetin glycosides), catechins and oligomeric procyanidins, as well as anthocyanins in red apples. Apples and apple juice are particularly good sources of oligomeric procyanidins (OPC) composed of (epi)catechin units which have recently gained interest because of potential health promoting effects. In apples OPC constitute 63-77% of all polyphenols (3). Several studies have shown higher cancer-preventive efficacy of cloudy apple juice in comparison to the clear product, which might be related to higher content of OPC (4, 5).

Apple OPC were found to inhibit colon cancer induced in rats by azoxymethane (6). This carcinogen similar to other N-nitroso compounds, including *N*-nitrosodiethylamine (NDEA), requires metabolic activation in order to exert tumorigenic activity. Metabolic activation is catalyzed by hepatic microsomal CYP450, mainly CYP2E1 and 2B (7).

In the case of N-nitrosoamines, including hepatocarcinogenic NDEA, cytochromes P450 mediated hydroxylation at position α to the nitroso group and

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formation of α-hydroxynitrosoamine have been considered a crucial step in the bioactivation to ultimate carcinogenic forms resulting in DNA alkylation, which might initiate the process of tumorigenesis. Moreover, it has been suggested that NDEA, besides being metabolized to reactive electrophiles, causes the generation of reactive oxygen species (ROS) leading to oxidative stress and cellular injury (8).

Phase II enzymes such as glutathione S-transferases (GSTs) conjugate activated phase I metabolites to endogenous ligands and enhance their detoxification and excretion. The reduction of elevated phase I enzymes activities to physiological levels and enhancing excretion of carcinogens *via* the upregulation of phase II enzymes are considered important chemoprevention strategies of cancers induced by exo- and endogenous carcinogens (3). This strategy refers also to apple juice.

Recently, Kujawska et al. (9) using the *in vivo* rat model with NDEA and carbon tetrachloride (CCl_4) challenge demonstrated a decrease in microsomal lipid peroxidation in the liver of rats treated with cloudy apple juice and NDEA in comparison to values obtained from animals given NDEA alone. The similar effect was observed in the case of CCl_4. However, DNA damage in the whole blood leukocytes was partially reduced only in animals treated with apple juice and NDEA but not in their counterparts exposed to juice and CCl_4.

Moreover, Soyalan et al. (10) described the elevation in the expression of the antioxidant response element-dependent genes in the distant colon of rats consuming cloudy apple juice, however, in the hepatic tissue, only NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione peroxidase were upregulated. The reported results showed that the effects of cloudy apple juice depend on metabolic pathway and tissue as well as on the kind of carcinogen tested.

The aim of our current study was to evaluate the effect of the long term treatment of rats with cloudy apple juice alone or in combination with NDEA on the phase I and II enzymes and hepatic DNA damage and the liver injury.

EXPERIMENTAL

Chemicals

NDEA, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, resorufin, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, p-nitrophenol, glutathione, 1-chloro-2,4-dinitrobenzene (CDNB), 2,6-dichlorophenolindophenol (DPIP), dicoumarol, NADP, NADPH, dithiothreitol, sucrose, low-melting point (LMP) agarose, bovine serum albumin, ethidium bromide and Tris were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Normal melting point agarose was Prona Plus Agarose, Triton X-100 was purchased from Park Scientific, (Northampton, UK). Primary and secondary antibodies against CYP1A1/1A2, β-actin, GST α, GST μ, GST π, GST θ and NQO1 were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary and secondary antibodies against CYP2E1 were supplied by Oxford Biomedical Research (Oxford, MI, USA). Primary and secondary antibodies against CYP2B were obtained from BD Biosciences (Woburn, MA, USA). All the antibodies used in these experiments were specific for their respective proteins, and according to the information provided by suppliers there was no cross-reactivity within the isozymes of the same family. Rainbow colored protein molecular weight marker was purchased from Amersham Pharmacia Biotechnology (Piscataway, NJ, USA). Commercial reagent kits for the determination of albumin, bilirubin, creatinine, blood urea nitrogen (BUN) and alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), sorbitol dehydrogenase (SDH), lactate dehydrogenase (LDH) and γ glutamyl transferase (GGT) activities were provided by Pointe Scientific, Inc. (Canton, MI, USA).

All the other chemicals were commercial products of the highest purity available.

Animals and treatments

Male Wistar rats (6 weeks of age), provided by the University of Medical Sciences, Department of Toxicology Breeding Facility (Poznań, Poland), were housed in polycarbonate cages ($30 \times 20 \times 25$ cm; $4 \leq$ rats/cage), containing hardwood chip bedding. Commercial rat food (Labofeed H, ISO 9001 certified) and distilled water were available without restriction. The experimental animals were randomly divided into four experimental groups each of six rats.

The animals were treated by gavage with 10 mL of apple juice per kg body weight for 28 consecutive days. The chosen juice dose corresponds to approximately 500-600 mL of juice consumed daily by an average-weight adult individual. On day 27, NDEA was administered *i.p.* in a single tumor-initiating dose of 150 mg/kg body weight (11). Control groups of animals received distilled water without restriction. All the experiments were conducted according to the European guidelines for the care and use of laboratory animals and were approved by the Regional Ethics Committee (No. 33/2007).

Cloudy apple juice preparation

Apples Shampion before processing were stored for 3 months at temperature +1.5°C in normal atmosphere until complete starch degradation measured by iodine test. Only sound fruits after storage were used for juices production. Before processing, apples were ground using Fryma perforated disc mill with disc having circular openings of 6 mm (BASIS 91/55, Fryma-Maschinen AG, Rheinfelden, Switzerland).

Cloudy juices were pressed using a rack and frame press (Bucher, Niederweningen, Switzerland), ascorbic acid was added during apples grinding (200 mg/kg of apples). Raw cloudy juice was centrifuged using continuous flow disk stack centrifuge (LAB 102B-25, Alfa Laval, Brentford, Middlesex, UK) at 1500 rpm and hot filled at 96-98°C into 0.25 L bottles using plate heat exchanger (P20-VB, Alfa-Laval Food Engineering, Lund, Sweden). After 30 min, bottles were cooled in tap water and then stored in the dark at 4°C for 4 weeks prior to experiments.

Determination of phenolic compounds in CAJ (12)

Qualitative characterization of cloudy apple juice was performed by HPLC analyses using a Phenomenex Fusion RP column (250 × 4.6 mm; 4 µm) with a guard column . The mobile phase consisted of 10.2% acetic acid in 2 mmol/L sodium acetate (solvent A) and acetonitrile (solvent B). The flow rate was kept constant at 0.5 mL/min for a total run time of 73 min at 25°C. The system was run with a gradient program: 3% B (0-20 min); 3-17% B (20 min); 17-40% B (25 min); 40-90% B (3 min); 90-90% B (4 min); and 90-0% B (1 min).

Phenolic content in natural cloudy apple juices was 230.1 mg/L. Main group consisted of flavan-3-ols and oligomeric procyanidins (Fig. 1) followed by phenolic acids. Dihydrochalcones (phloridzin and phloretin xyloglucoside) are very specific compounds of apple juice (13, 14). These compounds are present mainly in apple seeds and peel (15, 16) and during fast processing of fruits in to cloudy juices remain in the apple pomace.

Preparation of liver homogenates and cytosolic and microsomal fractions

Twenty four hours after the last treatment, the rats were anesthetized by ketamine and blood was collected by heart puncture into heparinized tubes and centrifuged (1000 × g for 10 min at 4°C) to separate plasma for the determination of albumin, bilirubin, cholesterol, creatinine, BUN levels and ALT, AST, SDH, LDH, GGT activities. The livers were removed, rinsed in ice-cold buffered 0.2 M sucrose (pH 7.5) and homogenized in the same medium. Cytosolic and microsomal fractions were prepared by differential centrifugation as described previously (17). Protein concentrations were determined by the method of Lowry et al. (18) using bovine serum albumin as the standard.

Phase I and II enzyme activity assays

The activities of ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and pentoxyresorufin-*O*-dependentylase (PROD) were measured as described previously (19, 20). The activity of *p*-nitrophenol hydroxylase (PNPH) was determined according to the Reinke and Moyer (21) protocol. Cytosolic NQO1 activity was assayed as described by Ernster (22) and modified by Benson et

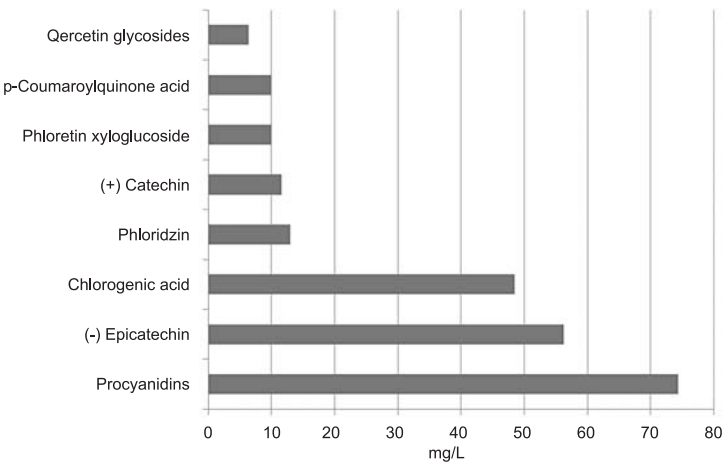

Figure 1. Composition of phenolic compounds in cloudy apple juice

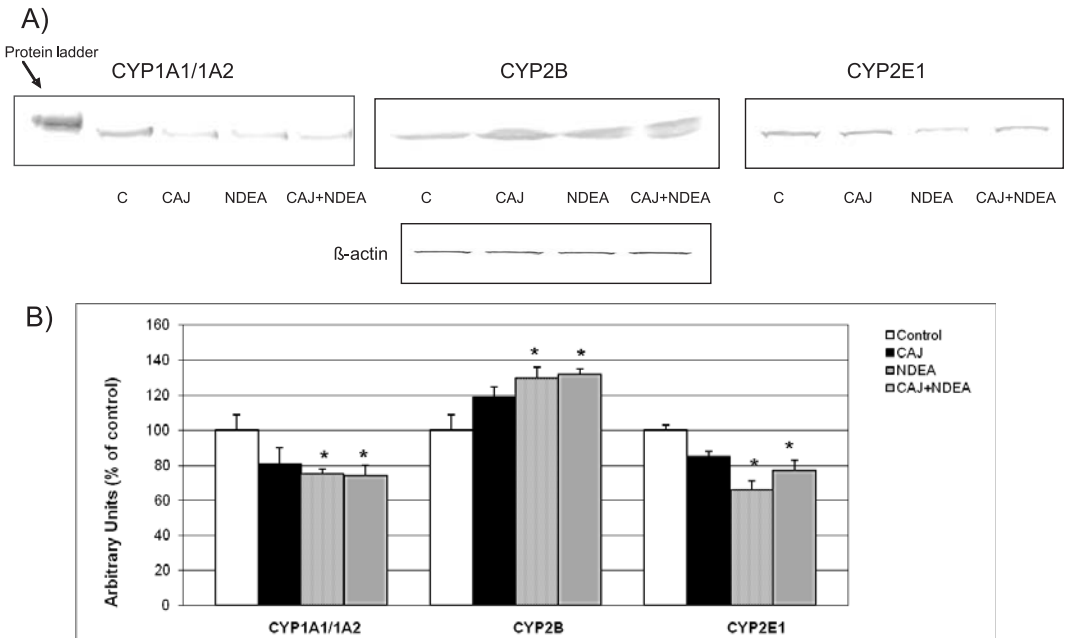

Figure 2. Effect of cloudy apple juice and NDEA on the expression of CYP1A1/1A2, CYP2B, CYP2E1 in microsomal fractions from rat liver. (**A**) Western blot analysis – representative blot is shown: C – control, CAJ – Cloudy apple juice, NDEA – N-nitrosodiethylamine, CAJ+NDEA – Cloudy apple juice + N-nitrosodiethylamine. The β-actin protein was used as an internal standard.(**B**) Data (mean ± SEM) present percentage of value obtained in control group, (expressed as arbitrary units), from 6 different animals per each experimental group (n = 6). Measurements were performed at least three times. Protein expression was quantified by densitometry analysis. Asterisk above bar denote statistically significant differences from * control group, $p < 0.05$

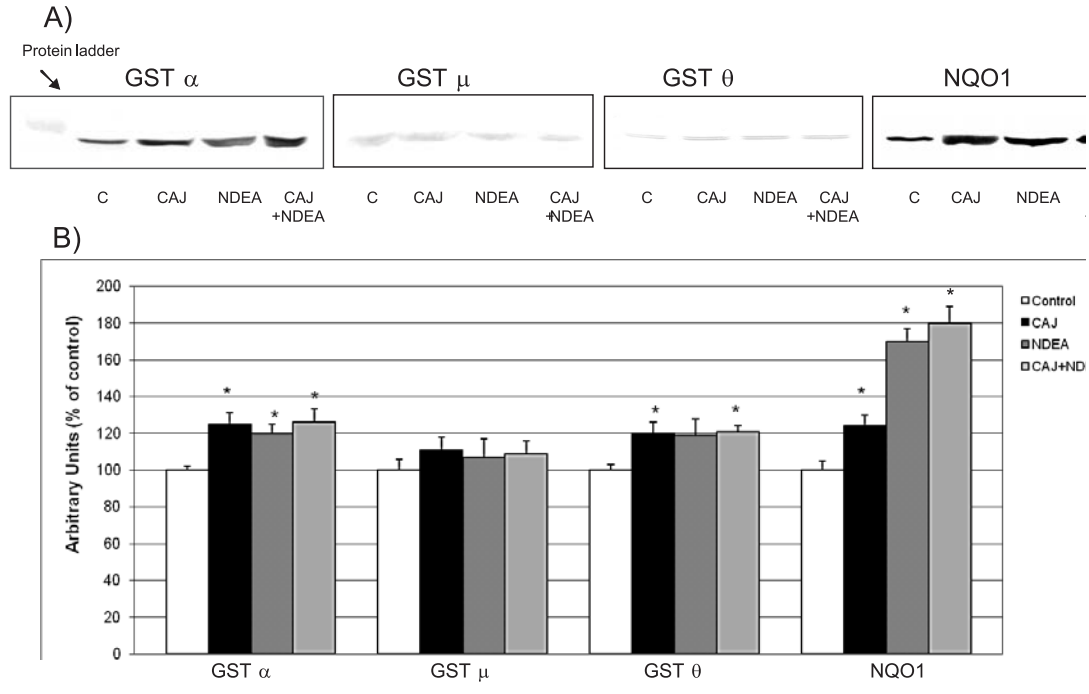

Figure 3. Effect of cloudy apple juice and NDEA on the expression of GST α , GST μ , GST π and NQO1 in cytosolic fractions from rat liver. (**A**) Western blot analysis – representative blot is shown: C – control, CAJ – Cloudy apple juice, NDEA – N-nitrosodiethylamine, CAJ+NDEA – Cloudy apple juice + N-nitrosodiethylamine.(**B**) Data (mean ± SEM) present percentage of value obtained in control group, (expressed as arbitrary units), from 6 different animals per each experimental group (n = 6). Measurements were performed at least three times. Protein expression was quantified by densitometry analysis. Asterisk above bar denote statistically significant differences from * control group, $p < 0.05$

al. (23) with NADPH as the electron donor and DPIP as the electron acceptor. The activity of GST was measured by the method of Habig et al. (24), using CDNB as a substrate.

Protein immunoblotting

Cytosolic and microsomal proteins (20-100 μg) were separated on 10 or 12% SDS-PAGE slab gels by the method of Laemmli (25). The proteins were transferred to nitrocellulose membranes using the method of Towbin et al. (26) and after blocking with 5% or 10% skimmed milk they were probed with mouse anti-rat CYP1A1/1A2, goat anti-rat CYP2B, goat anti-rabbit CYP2E1, rabbit anti-human GST α, goat anti-rat GST μ, rabbit anti-human GST π, mouse anti-human GST θ, goat anti-human NQO1 or rabbit anti-mouse β-actin antibodies. As the secondary antibodies in the staining reaction, the alkaline phosphatase-labeled anti-goat IgG, anti-mouse IgG or anti-rabbit IgG were used. The β-actin protein was used as an internal control. The amount of the immunoreactive product in each lane was determined by densitometric scanning using BioRad GS 710 Image Densitometer (BioRad Laboratories, Hercules, CA, USA). Values were calculated as relative absorbance units (RQ) per mg protein.

Comet assay

Single cell gel electrophoresis in alkaline conditions (pH > 13) was performed in the liver homogenates according to the method described by Hartmann et al. (27). Samples embedded in the LMP agarose were submitted to the procedures of cell lysis, DNA unwinding, electrophoresis and neutralization and then they were dehydrated in the absolute ethanol, dried and stored at room temperature, protected from light. Just before microscopic evaluation, the slides were rehydrated and stained with ethidium bromide (0.05 mg/mL). Images of comets were captured with a digital camera. For each sample 100 comets were scored. The comets were divided into 5 groups according to the degree of the DNA damage (28). A total damage score for each sample on the slide was calculated by multiplying the number of cells classified to each grade of damage by the numeric value of the grade and summing over all grades. The results obtained in the arbitrary units were expressed as the percentage of the values received in the control group.

Statistical analysis

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective con-

Table 1. Effect of cloudy apple juice and NDEA on the selected plasma enzymatic markers of rat liver function.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
ALT (IU/L)	35.12 ± 1.96	43.93 ± 1.81 (125)*	87.80 ± 2.80 (250)*	50.22 ± 2.09 (143)*,**
AST (IU/L)	63.24 ± 2. 11	59.44 ± 0.83 (94)	119.52 ± 2.20 (189)*	115.09 ± 2.56 (182)*
SDH (IU/L)	5.02 ± 0.41	4.03 ± 0.09 (80)*	30.67 ± 2.34 (611)*	25.04 ± 1.37 (499)*,**
LDH (IU/L)	189.80 ± 3.39	212.58 ± 4.83 (112)	265.72 ± 8.23 (140)*	216.37 ± 6.24 (114)
GGT (IU/L)	5.09 ± 1.01	6.27 ± 0.09 (123)*	9.11 ± 0.23 (179)*	7.53 ± 0.12 (148)*,**

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. Values in parentheses represent percent of control value. * Significantly different from control, $p < 0.05$. ** Significantly different from NDEA-treated rats, $p < 0.05$.

Table 2. Effect of cloudy apple juice and NDEA on the selected plasma biochemical markers of rat liver and kidney functions.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
Albumin (g/dL)	5.14 ± 0.09	5.12 ± 0.08 (100)	4.63 ± 0.11 (90)	5.10± 0.07 (99)
Bilirubin (mg/dL)	0.28 ± 0.04	0.29 ± 0.11 (104)	0.59 ± 0.06 (211)*	0.46±0.07 (164)*,**
Creatinine (mg/dL)	0.35 ± 0.04	0.38 ± 0.01 (109)	0.69 ± 0.05 (197)*	0.46±0.07 (131)*,**
BUN (mg/dL)	26.56 ± 0.21	26.83 ± 0.26 (101)	23.64 ± 0.21 (89)	24.87±0.31 (94)

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

trols was assessed by Tukey's *post hoc* test, with $p < 0.05$.

RESULTS

Selected biochemical markers of liver function in blood

The effects of CAJ and NDEA on selected liver function biochemical parameters (ALT, AST, ALP, SDH, LDH, GGT activities and albumin, bilirubin, creatinine, BUN) are presented in Tables 1 and 2. CAJ significantly increased ALT and GGT activities, but decreased SDH. Treatment of rats with a single dose of 150 mg/kg body weight of NDEA alone resulted in a statistically significant increase in the activity of all the tested enzymes in blood plasma in comparison to values from the control group (by 40-511%). Pretreatment with CAJ protected against the NDEA induced damage, reducing the activity of ALT, SDH, and GGT, and also bilirubin and creatinine levels.

Phase I enzymes in rat liver

The effects of CAJ and NDEA on cytochrome P450-dependent enzymes in rat liver are summarized in Table 3. Twenty eight days of forced feeding with cloudy apple juice alone significantly decreased the activities of MROD (the marker of CYP1A2) and PNPH (the marker of CYP2E1) by 19 and 20%, respectively, in comparison with the results from the control group of animals receiving water only. NDEA treatment reduced the activities of EROD (the marker of CYP1A1), MROD and PNPH by 40, 31 and 45%, respectively. The opposite effect was observed for PROD (the marker of CYP2B). As shown in Table 3, CAJ increased the activity of PROD by 25%, while NDEA enhanced it by 52%.

Pretreatment with CAJ further increased PROD activity elevated by NDEA treatment. Modulation of P450 enzymes activities was accompanied by changes in the relevant proteins levels. Western blot analysis with CYP2E1 and CYP1A1/1A2 specific antibodies (Fig. 2) revealed statistically significant decrease in the corresponding protein level in the NDEA-treated animals in comparison to the value achieved in the control rats. Densitometry of the bands presented in Figure 2 showed an enhanced level of CYP2B (by about 30%) in the liver of animals exposed to NDEA and the combined treatment with CAJ and NDEA.

Phase II enzymes in rats liver

The effects of CAJ administration alone or in combination with NDEA on phase II enzymes activities are presented in Table 4. Treatment with CAJ enhanced the activities of GST and NQO1. NDEA alone increased GST and NQO1 by 22 and 81%,

Table 3. Effect of cloudy apple juice and NDEA on the activity of cytochromes P450 in rat liver.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
EROD	35.14 ± 1.57	32.66 ± 1.48 (93)	21.08 ± 1.32 (60)*	23.54 ± 2.25 (67)*
MROD	31.76 ± 1.34	25.73 ± 0.86 (81)*	21.91 ± 0.55 (69)*	24.00 ± 0.9 (66)*
PROD	14.81 ± 0.88	18.05 ± 0.60 (125)*	22.51 ± 0.80 (152)*	25.77 ± 1.18 (174)*,**
PNPH	578.13 ± 54.83	462.50 ± 14.48 (80)*	319.08 ± 37.10 (55)*	384.88 ± 45.80 (60)*

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. EROD, MROD, PROD - pmol resorufin formed/min per mg of protein. PNPH - pmol p-nitrocatechol formed/min per mg of protein. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

Table 4. Effect of cloudy apple juice and NDEA on the activity of phase II enzymes in rat liver.

Treatment	Control	CAJ	NDEA	CAJ + NDEA
GST	992.95 ± 41.44	1191.54 ± 35.03 (120)*	1211.39 ± 50.02 (122)*	1320.62 ± 43.84 (133)*
NQO1	96.08 ± 6.91	126.34 ± 3.90 (131)*	173.90 ± 17.79 (181)*	201.77 ± 9.17 (210)*,**

Values are the means ± SEM from 6 animals. Each assay was run in triplicate. GST - nmol 1-chloro-2,4-dinitrobenzene conjugated formed/min per mg of protein. NQO1 - nmol 2,6-dichloroindophenol reduced/min per mg of protein. Values in parentheses represent percent of control value. *Significantly different from control, $p < 0.05$. **Significantly different from NDEA-treated rats, $p < 0.05$.

Table 5. Effect of cloudy apple juice and NDEA on the extent of DNA damage in homogenates of rat liver.

Treatment	DNA damage
Control	77.7 ± 3.19
CAJ	87.3 ± 2.36 (113)*
NDEA	123.7 ± 3.38 (159)*
CAJ + NDEA	120.0 ± 0.817 (154)*

Values are the means ± SEM from 6 animals. Each assay was run in duplicate. DNA damage in homogenates of rat liver in arbitrary units. Values in parentheses represent percent of control value.
* Significantly different from control, $p < 0.05$.

respectively. A similar effect was also observed in animals after the combined treatment with CAJ and NDEA with the exception of NQO1 which activity was further increased.

Figure 3 presents the immunoblots of GST isozymes and NQO1 and their quantitative analysis. GST π protein was not detected in the liver. CAJ increased the constitutive expression of GST μ, α and θ (by 11, 25 and 20%, respectively), but did not affect the NDEA induced GST proteins. The increased activity of NQO1 was accompanied by the elevated level of the enzyme protein as a result of cloudy apple juice or NDEA treatment as well. The pretreatment with CAJ did not significantly affect the level of NDEA-induced NQO1 protein.

Comet assay analysis of DNA damage in the liver homogenates

The effects of CAJ and NDEA treatments on DNA damage are presented in Table 5. CAJ administered to rats for 28 days moderately augmented the scale of DNA damage in the liver. NDEA alone increased the extent of DNA lesions by ~59%. Pretreatment of rats with CAJ did not significantly reduce DNA damage in NDEA-administered animals.

DISCUSSION

Several epidemiological studies suggest that fruits and vegetables in basal diet afford a significant protection against a wide range of common human cancers (29, 30). Some of the surveys showed that the protective effect of a high intake of fruits and vegetables was only weakly correlated with single dietary constituents or the distinct groups of phytochemicals. Moreover, cancer intervention studies with a single compound or simple combinations of promising anticarcinogens, often failed to show a cancer protective effect against several cancer types. These data indicate that the protective action most likely arise from the combined intake of several dietary components such as fruit or vegetables juices, rather than being a result of a single anticarcinogenic components (31, 32).

Apples and apple products, including juices and extracts, were included in health-related studies around the world due to their rich content of diverse phytochemicals, particularly specific classes of polyphenols. Although apple products were shown to exert beneficial effect in numerous pathologies, their preventive activity against chemically induced carcinogenesis was relatively less explored. Oszmiański et al. (33) found markedly higher content of procyanidins and pectins in cloudy apple juices, which was associated with higher radical-scavenging and antioxidant capacities. Additionally, Barth et al. (4, 5) demonstrated higher cancer-preventive efficacy of cloudy apple juice in comparison with clear juice. The authors suggested that these juices could modulate biochemical pathways by antagonistic, additive and/or synergic mechanisms.
In the present study, we focused on the evaluation of a possible interference of CAJ with NDEA-induced effects. This chemical is a very potent carcinogen that induces liver carcinomas and gastrointestinal tract neoplasms in rats (34). A prominent phenomenon during hepatocarcinogenesis is an alteration of the expression of drug-metabolizing enzymes. In the case of NDEA, cytochromes P450 CYP2E1- and 2B-mediated activation leads to the formation of electrophiles, which ethylate DNA and, if not repaired, to mutation. Moreover, the uncoupling of electron transfer and oxygen reduction from monooxygenation by CYP2B1 and CYP2E1 could result in the release of $O_2^{\cdot-}$ and H_2O_2 and cause liver injury (8, 35).

The results of our present study confirm our previous observations concerning NDEA effects in rat liver (36, 37).

The treatment with this compound resulted in an increase in hepatic DNA and liver tissues damage biomarkers, and the reduction in EROD, MROD and PNPH activities. At the same time NDEA induced CYP2B and phase II enzymes, GST and NQO1.

Twenty eight days administration of CAJ resulted in a decrease of MROD and PNPH activities. The expression of P450 isozymes, CYP1A1/A2, CYP2B and CYP2E1 was not changed in comparison with the values found in control group of animals. Since these P450 isoforms are involved in the activation of several classes of chemical carcinogens including polycyclic aromatic hydrocarbons and nitrosamines, their inhibition is

expected to block both the toxicity and carcinogenicity of these compounds. Such mechanisms of anticarcinogenic activity was proposed for several phytochemicals including organosulfur compounds and coumarins (38, 39). Decreased expression and activity of CYP1A as a result of apple juice extracts treatment was observed also by Pohl et al. (40) in Caco-2 colon cancer cells. Moreover, feeding with CAJ in our study increased the activity and protein level of phase II enzyme NQO1 and, to a lesser extent, of GST. Thus, the inhibition of CYP2E1 in concert with the induction of NQO1 might contribute to the potential anticarcinogenic activity of CAJ. We suggested a similar mechanism for chokeberry juice (36). Chokeberry juice like cloudy apples juice contains high amount of OPC. Since these compounds were shown to be responsible for the high antioxidant activity of CAJ, they might be responsible also for the effects observed in our current study.

On the other hand, CAJ slightly increased the activity and expression of CYP2B. This is a large P450 subfamily that encodes versatile catalysts of xenobiotics and steroid hydroxylation. Some of them induce phenobarbital-type response, epoxide hydrolase and GST (41). Thus, the induction of CYP2B may be related to tumorigenesis promotion, but also the enhancement of the detoxification of carcinogens.

Although CAJ feeding only slightly increased total GST activity, immunoblotting revealed an increase of GST α and θ. Increased level of GST α might be beneficial as this isoform contributes to the protection against ROS and the products of lipid peroxidation such as 4-hydroxynonenal (42), while GST θ class 1 and 2 (GSTT1 and GSTT2) react with a wide range of xenobiotics, including nitrosamines, what implicates their possible role in the prevention of carcinogenesis induced by NDEA. The possible involvement of GSTT2 in the anticarcinogenic effect of apples further supports the observation of Petermann et al. (43) that the induction of this GST isoform by apple polyphenols protects colon epithelial cells against genotoxic damage.

Consistently with our previous and the other authors observations (44, 45), the GST π protein was not detected in the liver, since this isoform is present in placental liver only, but not in adult rats (46). Its induction requires the application of two-stage carcinogenesis protocol in which initiation achieved with NDEA application is followed by treatment with promoter or partial hepatectomy (47).

Moreover, our recent study showed that phloretamide, one of apple components, activates the Nrf2/ARE pathway in human hepatocytes in culture (48). Thus, it is possible that this mechanism is also responsible for the induction of NQO1 and GST by apple juice in our current study.

Pretreatment with CAJ before the administration of NDEA reduced ALT, SDH and GGT activities and plasma bilirubin and creatinine levels. CAJ treatment alone caused minor, however significant rise in DNA damage, whereas NDEA induced larger DNA lesions. CAJ pretreatment before NDEA challenge did not protect hepatic DNA from decomposition. Among liver damage biomarkers, the reduction in SDH activity, which was also observed after feeding with CAJ only, seems to be the most interesting phenomenon.

Apples were identified as the unique flavonoid-rich food that might be protective against type 2 diabetes (49). SDH is a key enzyme in the polyol pathway converting sorbitol to fructose which level is significantly elevated in diabetes. The reduction in SDH activity may suggest that the antidiabetic effect of apples may be related to improvement of the polyol pathway as was shown for ursolic acid (50).

In our present experiment, CAJ pretreatment did not affect the expression and activity of CYPs increased by NDEA exposure, except for CYP2B which was elevated. Since, as it was mentioned above, the consequences of this CYP450 subfamily induction are complex, the explanation of this observation requires additional studies.

The most marked effect of NDEA on cytochrome P450 dependent enzymes (EROD, MROD, PNPH) was observed both on the levels of the enzymes activity and respective CYP proteins (CYP1A1/CYP1A2, CYP2E1), there was no significant correlation between the CYPs protein level and enzyme activities in the case of CAJ.

CAJ pretreatment further increased the expression and activity of NQO1 induced by NDEA. This enzyme is generally assumed to possess important protective properties, both by detoxifying carcinogenic compounds as well as by preventing the generation of oxygen radicals (51).

Collectively, the results of our present study indicate that metabolic alterations induced by cloudy apple juice may protect against liver damage and attenuate the effect of chemical carcinogens. Moreover, CAJ treatment decreased sorbitol dehydrogenase activity, a key enzyme of sorbitol pathway, particularly active in diabetic patients. Since cloudy apple juice is one of the most common diet components the results of our current study, although require further investigations, provide

rationale for its recommendation as chemopreventive food item.

Declaration of interest

The authors declare no conflicts of interest. All authors approved the final version submitted for publication.

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DRUG SYNTHESIS

1-[(IMIDAZOLIN-2-YL)AMINO]INDOLINE AND 1-[(IMIDAZOLIN-2-YL)AMINO]1,2,3,4-TETRAHYDROQUINOLINE DERIVATIVES: NEW INSIGHTS INTO THEIR CIRCULATORY ACTIVITIES

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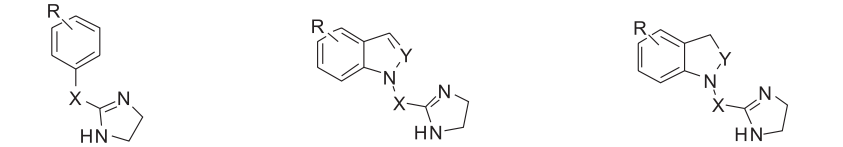
Abstract: *N*-[(Imidazolin-2-yl)amino]indolines and *N*-[(imidazolin-2-yl)amino]-1,2,3,4-tetrahydroquinolines, previously described in patent literature as hypertensive agents, were synthesized and tested *in vitro* for their affinities to α_1- and α_2-adrenoceptors as well as imidazoline I_1 and I_2 receptors. The compounds most potent at either α_1- or α_2-adrenoceptors were administered intravenously to normotensive Wistar rats to determine their effects on mean arterial blood pressure and heart rate. Upon intravenous administration at dose of 0.1 mg/kg to normotensive male Wistar rats, the initial transient pressor effect was followed by long-lasting hypotension and bradycardia. In view of the above results the 1-[(imidazolin-2-yl)amino]indolines and [(imidazolin-2-yl)amino]-1,2,3,4-tetrahydroquinolines are now found to possess circulatory profile characteristic of the centrally acting clonidine-like hypotensive imidazolines.

Keywords: imidazolines, indolines, 1,2,3,4-tetrahydroisoquinolines, α-adrenoceptors, imidazoline receptors, hypertensive effect, hypotensive effect

Imidazoline-containing agents acting at α_2-adrenoceptors exhibit important pharmacological effects including hypotension, bradycardia, analgesia, sedation, mydriasis, organ-protection, stimulation of growth hormone secretion and decreased output of endocrine and exocrine secretory glands, such as decreased insulin secretion and decreased salivation (1-11). On the other hand, the therapeutic potential of agents which selectively interact with α_1-adrenoceptors includes nasal congestion, urinary incontinence as well as sexual, CNS and eating dysfunctions (12-16).

It is well established that imidazoline derivatives of type **A** with methylene bridge between the imidazoline and the aryl ring (Figure 1, X = CH_2) such as xylometazoline, oxymetazoline and naphazoline induce an increase in blood pressure due to peripheral α_1-adrenergic receptor stimulation, while



A, R = alkyl, halogen
X = NH (**hypotensive**)
X = CH_2 (**hypertensive**)

B, R = alkyl, halogen
Y = N, CH
X = NH, CH_2 **(hypotensive)**

C, R = alkyl, halogen
Y = $(CH_2)_n$
X = NH (**hypertensive?)**

ref. (36,46) – *marsanidine*, *7-Me-marsanidine*

Figure 1. Imidazoline derivatives with circulatory activity

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the analogues related to clonidine with amino bridge (Figure 1, X = NH) cause a secondary long lasting decrease in blood pressure caused by central α_2-adrenoceptor activation (17). Our previous investigations on α-adrenoceptor ligands led to the discovery of imidazoline-containing indoles and indazoles (Figure 1, structure **B**) which, regardless the structure of bridging moiety X (either NH or CH_2), exhibited hypotensive activity due to either α_2-adrenoceptor agonist or α_1-adrenoceptor antagonist activity (18-22). However, a comprehensive survey of patent literature revealed that imidazolines connected to the partially hydrogenated indole and quinoline rings *via* NH bridge (Figure 1, structure **C**) were described as hypertensive agents when administered to rats at doses as low as 0.02-0.5 μg/kg (23, 24). Although imidazolines represent rather peculiar class of adrenergic agents because small structural modifications may result in altering the balance between agonist and antagonist activity (25), from the point of view of structure-activity relationships (SAR), the results of biological tests presented in aforementioned patents seemed to be rather dubious, since the overall structure of compounds **C** bears resemblance to the hypotensive amine-bridged imidazolines of type **B** (Fig. 2). Molecular modeling studies with use of Spartan 08 program v. 1.2 indicate that the imidazoline N1 nitrogen atoms in **C** and **B** are situated 5.89 Å and 6.07 Å, respectively, apart from phenyl ring centroid and lie at the distance of 1.27 Å and 1.69 Å, respectively, from the phenyl ring best plane. Therefore, in the present work, the influence of partial hydrogenation of **B** leading to indolines and tetrahydroquinoline analogues of type **C** on α-adrenoceptor affinity and selectivity has been explored. The hemodynamic effects of such ligand modification in anesthetized rats were also reinvestigated.

EXPERIMENTAL

Melting points were determined on a Boetius apparatus and are uncorrected. FT-IR spectra were measured on Nicolet 380 apparatus. Results of C, H, N elemental analyses were within ±0.4% of theoretical values. ^{1}H- and ^{13}C-NMR spectra were recorded on Varian Gemini 200 or Varian Unity 500 apparatus. ^{1}H and ^{13}C chemical shifts were measured relative to the residual solvent signal at 2.50 ppm and 39.5 ppm (DMSO-d_6) or 7.26 and 77.2 ($CDCl_3$). The following compounds were obtained according to previously described procedures: 8-methyl-1,2,3,4-tetrahydroquinoline (26), 4-chloroindoline (27), *N*-amino-indolines and *N*-amino-1,2,3,4-tetrahydroquinolines (28), 2-chloro-4,5-dihydro-1*H*-imidazole (29), *N*-*tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole (30). Structure optimization of indole (**B**) and indoline (**C**) was carried out using Spartan program v. 8.0 (Wavefunction Inc., Irvine, CA, USA).

N-nitroso-indolines 2a-d and *N*-nitroso-1,2,3,4-tetrahydroquinolines 2a,b

To the appropriate cyclic amine (20 mmol) in hexane (15 mL) amyl nitrite (60 mmol, 7 g, 8 mL) was added in one portion. The resulting mixture was

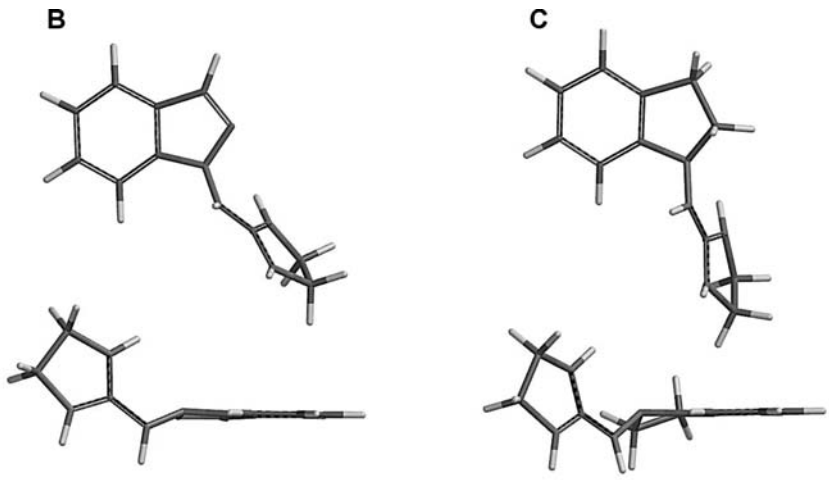


Figure 2. Minimum energy conformations of indazole derivative **B** (*left*) and indoline derivative **C** (*right*) optimized at density functional level of theory (B3LYP) with the 6-31G* basis set.

stirred for 30 min at room temperature. The precipitated solid was collected by filtration, washed with hexane, dried and used for preparation of corresponding *N*-aminoindolines **3a-d** and *N*-amino-1,2,3,4-tertahydroquinolines **8a**,**b** without purification.

***N*-aminoindolines 3a-d and *N*-amino-1,2,3,4-tetrahydroquinolines 8a,b**

To a suspension of $LiAlH_4$ (32 mmol, 1.12 g) in THF or anhydrous diethyl ether (50 mL) was added dropwise a solution of appropriate *N*-nitrosoamine (21.2 mmol) in anhydrous diethyl ether/THF (9 : 1, v/v) (100 mL) and the resulting mixture was stirred at room temperature for 30 min and then at reflux for 2 h. Upon cooling to 0°C water was added dropwise to destroy excess $LiAlH_4$. The reaction mixture was filtered under reduced pressure and the filter cake was washed with dichloromethane. The combined filtrates were dried over $MgSO_4$ and concentrated to dryness under reduced pressure. *N*-aminoindoline **3d** and *N*-amino-1,2,3,4-tetrahydroquinoline **8b** thus obtained were converted to the corresponding hydrochlorides, while free bases **3a-c** and **8a** were used for further reaction without purification.

***N*-amino-4-chloroindoline hydrochloride (3d)**

Yield: 3.5 g (80%); m.p. 205–208°C. IR (KBr, cm^{-1}): 3044, 2900, 2680, 2513, 1605, 1575, 1540, 1454, 1075, 879, 824, 767, 705, 598; ^{1}H NMR (200 MHz, DMSO-d_6, δ, ppm): 10.69 (br. s, 3H, NH_3^+), 7.30–721 (m, 2H), 7.09–7.02 (m, 1H), 3.64 (t, 2H, *J* = 7.8 Hz), 3.02 (t, 2H, *J* = 7.8 Hz). ^{13}C NMR (50 MHz, DMSO-d_6, δ, ppm): 151.1, 129.9, 129.7, 127.6, 122.8, 110.9, 55.4, 27.1.

***N*-amino-8-methyl-1,2,3,4-tetrahydroquinoline hydrochloride (8b)**

Yield: 3.2 g (77%); m.p. 178–181°C. IR (KBr, cm^{-1}): 2926, 2830, 2744, 2660, 1596, 1578, 1522, 1458, 769; ^{1}H NMR (200 MHz, DMSO-d_6, δ, ppm): 10.0 (br. s, 3H NH_3^+), 7.13–7.02 (m, 3H), 3.43–3.38 (m, 2H), 2.83 (t, 2H, *J* = 6.7 Hz), 2.39 (s, 3H), 2.03–1.98 (m, 2H). ^{13}C NMR (50 MHz, DMSO-d_6, δ, ppm): 141.4, 134.7, 131.7, 128.8, 127.9, 126.9, 50.7, 25.8, 17.6, 15.8.

***N*-[(imidazolin-2-yl)amino]indolines (4a–d) and *N*-[(imidazolin-2-yl)amino-1,2,3,4-tetra-hydroquinolines (9a,b) and corresponding hydrochlorides 5a–d and 10a,b.**

Method A: To a stirred solution of 2-chloro-4,5-dihydro-1*H*-imidazole (25 mmol) in dichloromethane (30 mL) the corresponding *N*-aminoindoline (17 mmol) was added and the mixture was stirred for 24 h at room temperature. The precipitated solid was collected by filtration under reduced pressure. The crude product in the form of hydrochloride was dissolved in water and washed with dichloromethane to remove impurities. The aqueous phase was made alkaline (pH~10) with 5% aqueous NaOH and extracted with dichloromethane. The combined organic extracts were dried over $MgSO_4$ and evaporated to dryness to obtain the product in the form of free base as a white solid. The free base was dissolved in methylene chloride (5 mL) and the 3.2 M solution of hydrochloride in diethyl ether (3 mL) was added. The precipitated hydrochloride salt was collected by filtration.

According to the above procedure the following compounds were obtained:

***N*-[(imidazolin-2-yl)amino]indoline (4a)**

Yield 49%, m.p. 97–99°C. IR (KBr, cm^{-1}): 3169, 2930, 2872, 1628, 1595, 1496, 1284, 750. ^{1}H-NMR (200 MHz, DMSO-d_6, δ, ppm): 7.89 (d, *J* = 8.2 Hz 1H), 7.00–7.13 (m, 2H), 6.77 (t, 1H), 5.99 (s, 2H, NH), 3.91 (t, 2H, CH_2), 3.46 (s, 4H, CH_2), 3.06 (t, 2H, CH_2). ^{13}C-NMR (50 MHz, DMSO-d_6, δ, ppm): 159.08, 144.81, 130.72, 126.99, 124.52, 120.46, 113.75, 48.60 (2C), 27.46.

***N*-[(imidazolin-2-yl)amino]indoline hydrochloride (5a)**

Yield 98%, m.p. 323–326°C. IR (KBr, cm^{-1}): 3227, 3095, 1640, 1591, 1550, 1496, 1287, 1067, 761. ^{1}H-NMR (200 MHz, DMSO-d_6, δ, ppm): 8.79 (s, 2H, NH), 7.24–7.42 (m, 3H), 7.12 (t, 1H), 4.09 (t, 2H, CH_2), 3.75 (s, 4H, CH_2), 3.22 (t, 2H, CH_2),

***N*-[(imidazolidin-2-yl)imino]-2-methylindoline (4b)**

Yield: 30%; m.p. 53–55°C. IR (KBr, cm^{-1}): 3411, 3165, 3044; 2960, 2862; 1656 (C=N), 1604 (δ N-H), 1473, 1458, 1279, 1248, 750. ^{1}H NMR (200 MHz, $CDCl_3$, δ, ppm): 7.03–7.10 (m, 2H), 6.69–6.77 (m, 1H), 6.52–6.56 (m, 1H), 5.28 (br. s, 2H, 2 ´ NH), 3.42 (s, 4H), 3.04 (dd,1H, J_1 = 14.9 Hz, J_2 = 7.0 Hz), 2.52 (dd, 1H, J_1 = 14.9 Hz, J_2 = 11.4 Hz), 1.29 (d, 3H, *J* = 7.0 Hz). ^{13}C NMR (50 MHz $CDCl_3$, δ, ppm): 166.65 (C=N), 153.76, 128.57, 126.93, 123.70, 119.31, 109.90, 65.75, 42.42, 36.15, 18.96.

***N*-[(imidazolin-2-yl)amino]-2-methylindoline hydrochloride (5b)**

Yield: 100%; m.p. 234–236°C (lit. (23) m.p. 226–228°C); IR (KBr, cm^{-1}): 3400, 3250, 3142,

2966, 2902, 1679, 1600, 1477, 1459, 1286, 1069, 750, 670. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm): 10.39 (br. s, 1H, NH), 8.85 (br. s 1H, NH), 8.47 (br. s 1H, NH), 7.14–7.18 (m, 2H), 6.91 (t, 1H, J = 7.8 Hz), 6.69 (d, 1H, J = 7.8 Hz), 3.63–3.71 (m, 5H), 3.15 (dd, 1H, J_1 = 15.6 Hz, J_2 = 7.8 Hz), 2.56 (m, 2H), 1.35 (d, 3H, J = 7.8 Hz).

***N*-[(imidazolin-2-yl)amino]-1,2,3,4-tetrahydroquinoline (9a)**

Yield: 58%; m.p. 153–154°C. IR (KBr, cm^{-1}): 3405, 3161, 2947, 2856, 1654, 1489, 1451, 1302, 1275, 741. ^{1}H-NMR (200 MHz, DMSO-d_6, δ, ppm): 6.79–6.91 (m, 2H), 6.61–6.66 (m, 1H), 6.42–6.50 (m, 1H), 6.14 (s, 2H, NH), 3.27 (s, 4H, CH_2), 3.05 (t, 2H, CH_2), 2.67 (t, 2H, CH_2), 1.94–2.06 (m, 2H, CH_2). ^{13}C-NMR (50 MHz, DMSO-d_6, δ, ppm): 165.88, 148.99, 128.08, 126.23, 122.39, 116.58, 113.88, 50.93, 42.38 (2 C), 27.02, 22.35.

***N*-[(imidazolin-2-yl)amino]-1,2,3,4-tetrahydroquinoline hydrochloride (10a)**

Yield: 49%; m.p. 269–272°C. IR (KBr, cm^{-1}): 3113, 2930, 2890, 1661, 1606, 1452, 1284, 752. ^{1}H-NMR (200 MHz, DMSO-d_6, δ, ppm): 8.76 (s, 2H, NH), 7.02–7.13 (m, 2H), 6.70–6.84 (m, 2H), 3.67 (s, 4H, CH_2), 3.34 (s, 2H, CH_2), 2.71 (s, 2H, CH_2), 2.01 (s, 2H, CH_2).

Method B: A suspension of the appropriate *N*-aminoindoline or N-amino-1,2,3,4-tetrahydroquinoline (12.8 mmol) and *N-tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole (3.32 g, 15.36 mmol) in acetic acid (8 mL) was stirred at 60°C (oil bath) for 16 h and then the solvent was evaporated under reduced pressure. The viscous residue was treated with water (7 mL) and to the resulting mixture was added dropwise 5% aqueous NaOH to pH 9.5–10. The resulting mixture was extracted with dichoromethane (3 × 20 mL). The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. The oily residue was purified by chromatography on silica gel eluting with ethyl acetate and then ethyl acetate/methanol/triethylamine (8 : 1 : 1, v/v/v). The resulting *N*-[(imidazolin-2-yl)amino]amine free bases were transformed into the corresponding hydrochloride salts by treating with 3.2 M solution of hydrochloride in diethyl ether.

According to the above procedure the following compounds were obtained:

The free base **4c** was obtained as a viscous oil, and therefore, it was transformed into hydrochloride **5c** without characterization.

N-[(imidazolin-2-yl)amino]-7-methylindoline hydrochloride (5c)

Yield: 7%; m.p. 112–115°C (lit. (24) m.p. 194–196°C). IR (KBr, cm^{-1}): 3145, 2966, 2908, 2865; 1665; 1604. ^{1}H NMR (200 MHz, DMSO-d_6, δ, ppm): 10.54 (s, 1H, NH), 8.98 (s, 1H, NH), 8.37 (s, 1H, NH), 7.06 (d, 1H, J = 7.3), 6.95 (d, 1H, J = 7.8), 6.88 (dd, 1H, J_1 = 7.3, J_2 = 7.8 Hz), 3.87–3.83 (m, 1H), 3.68 (br. s, 4H), 3.27–3.21 (m, 1H), 3.15–3.10 (m, 1H), 2.82–2.75 (m, 1H), 2.17 (s, 3H). ^{13}C NMR (50 MHz, DMSO-d_6, δ, ppm): 160.8 (C=N), 147.6, 130.1, 129.2, 122.89, 122.85, 57.0, 43.3, 42.2, 27.4, 16.7.

***N*-[(imidazolin-2-yl)amino]-4-chloroindoline (4d)**

Yield 20%; m.p. 174–176°C. IR (KBr, cm^{-1}): 3388, 3162; 2857, 1650; 1599, 1451, 1279, 1262, 1109, 768. ^{1}H NMR (200 MHz, $CDCl_3$, δ, ppm): 6.99 (t, 1H, J = 7.7 Hz), 6.71 (d, 1H, J = 8.1 Hz), 6.49 (d, 1H, J = 7.7 Hz), 5.26 (br. s, 2H, NH), 3.43 (s, 4H), 3.38 (t, 2H, J = 7.7 Hz), 2.93 (t, 2H, J = 7.7 Hz). ^{13}C NMR (50 MHz, $CDCl_3$, δ, ppm): 166.2, 155.5, 130.0, 128.7, 127.2, 119.5, 108.6, 56.8, 42.7, 27.4.

***N*-[(imidazolin-2-yl)amino]-4-chloroindoline hydrochloride (5d)**

Yield 100%; m.p. 235–238°C. IR (KBr, cm^{-1}): 3082, 3030, 2871, 2798, 2719, 1664, 1604, 1589, 1449, 1261, 1129, 884, 771; ^{1}H NMR (200 MHz, DMSO-d_6, δ, ppm): 10.75 (s, 1H, NH), 8.79 (br. s, 2H, 2×NH), 7.18 (t, 1H, J_1 = 8.0 Hz), 6.95 (d, 1H, J = 8.0 Hz), 6.66 (d, 1H, J = 8.0 Hz), 3.80–3.50 (m, 6H), 3.10–2.85 (m, 2H).

The free base **9b** was obtained as a viscous oil which was transformed into hydrochloride **10b** without characterization.

***N*-[(imidazolin-2-yl)amino]-1,2,3,4-tetrahydro-8-methylquinoline hydrochloride (10b).**

Yield 20%; m.p. 248–251°C. IR (KBr, cm^{-1}): 3265, 3133, 3069, 3033, 3012, 2955, 2929, 2854, 1657, 1482, 1460, 1441, 1274, 1102, 764. ^{1}H NMR (200 MHz DMSO-d_6, δ, ppm): 10.53 (s, 1H, NH), 9.03 (s, 1H, NH), 7.92 (s, 1H, NH), 7.27–6.96 (m, 3H), 3.66 (s, 4H), 3.27–3.24 (m, 1H), 3.15–3.13 (m, 1H), 2.79–2.74 (m, 2H), 2.12 (s, 3H), 2.00–1.92 (m, 1H), 1.76–1.70 (m, 1H). ^{13}C NMR (50 MHz, DMSO-d_6, δ, ppm): 159.4 (C=N), 142.3, 132.6, 129.3, 128.8, 127.8, 124.3, 53.4, 43.1, 42.4, 26.7, 17.2, 17.8;

Radioligand binding assays

I_1-Binding site assay

Kidneys were obtained *post mortem* from male Sprague–Dawley rats (250–280 g) and crude P_2

membranes were prepared according to the methods of Lione et al. (31). Binding of [^{3}H]clonidine (3 nM, PerkinElmer) was investigated in the presence of 10 mM rauwolscine to preclude radioligand binding to α_2-adrenoceptors. The specific component was defined by 10 mM rilmenidine; under these conditions, the site labelled represents a model of the central I_1 binding site (32). Membrane aliquots (400 µL, 0.2–0.5 mg protein) were incubated with 11 concentrations of the test compounds over the range 0.1 nM – 100 µM. Incubations were carried out in 50 mM Tris-HCl buffer (pH 7.4) at room temperature for 45 min. Bound radioligand and free radioactivity were separated by rapid filtration through pre-soaked (0.5% polyethyleneimine) glass-fibre filters (Whatman GFB). Trapped radioligand was determined by liquid scintillation counting and the data were analyzed with GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, USA) to yield IC_{50} values (the concentration of tested ligand that displaces 50% of specifically bound [^{3}H]clonidine).

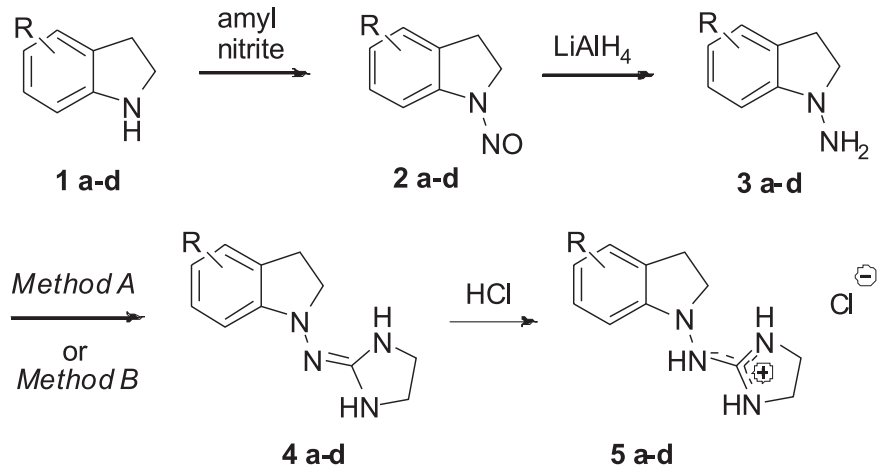
α_1- and α_2-adrenoceptor and I_2-receptor binding assays

Brains were obtained *post mortem* from male Sprague–Dawley rats (250–280 g) and crude P_2 membranes were prepared (31). Membrane aliquots (400 µL, 0.2–0.3 mg protein) were incubated with 11 concentrations of the tested compounds over the range 0.1 nM – 100 µM in the presence of the selective I_2 binding site radioligand [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline) (32) (1 nM), the α_1-adrenoceptor antagonist radioligand [^{3}H]prazosin (1 nM) or the α_2-adrenoceptor antagonist radioligand [^{3}H]RX821002 (2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1*H*-imidazole) (33) (1 nM) in a final volume of 500 µL. Non-specific binding was determined using 10 µM BU224 (2-(4,5-dihydroimidazol-2-yl)quinoline) (34) for I_2 binding, 10 µM phenylephrine for α_1-adrenoceptors and 10 µM rauwolscine to define α_2-adrenoceptor binding. Incubations were performed in triplicate at room temperature and were allowed to reach equilibrium (45 min). Bound and free radioactivity were separated by rapid filtration through pre-soaked (0.5% polyethyleneimine) glass-fibre filters (Whatman GF/B). Filters were then washed twice with 5 mL of ice-cold buffer and membrane-bound radioactivity remaining on the filters was determined by liquid scintillation counting. The data were analyzed by iterative non-linear regression curve fitting procedures with GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA). Each experiment was analyzed individually and equilibrium dissociation constants (K_i) were determined by the method of Cheng and Prusoff (35). The resulting values are given as the means ± SEM of three or four separate experiments.

***In vivo* studies: mean arterial blood pressure (MAP) and heart rate (HR) in rats**

Male Wistar rats, weighing 200-290 g, were purchased from the Animal House of the Medical University of Gdańsk, Poland. All *in vivo* experiments were approved by the Local Ethical Committee on Animal Experiments. The animals were fed a commercial rodent chow (Labofeed–B, Poland). Tap water was available *ad libitum*. Rats were anesthetized by *i.p.* injection of thiopental (Sandoz, Austria) at a dose of 70 mg/kg body weight and maintained under anesthesia by thiopental supplementation (30 µg/kg/min) during the experiment. The animals were placed on a heated table, and body temperature was maintained between 36 and 37°C. Tracheostomy was performed. Catheters were inserted into the carotid artery for blood pressure and heart rate monitoring, into a jugular vein for infusions, and into the bladder for free diuresis. After all surgical procedures, a 40 min recovery period was allowed to establish steady state. The rats were infused with isotonic saline (Fresenius Kabi, Poland) supplemented with thiopental at a rate of 1.2 mL/h. After 40 min of saline infusion, the tested compounds were administered as a 100 µL bolus through the venous catheter at a dose of 0.1 mg/kg. The time of administration of the compound was assumed as "time 0". Mean arterial blood pressure (MAP) and heart rate (HR) were monitored directly and sampled continuously at 100 Hz, as described previously (36) using Biopac Systems, Inc., Model MP 100 (Goleta, CA, USA). The results of recordings were elaborated with the help of the ACQKnowledge (Goleta, CA, USA) analysis system and were selected, scaled and filtered to remove signal disturbances. The recorded time domain transient data are presented as graphs with the help of Excel (Microsoft, USA).

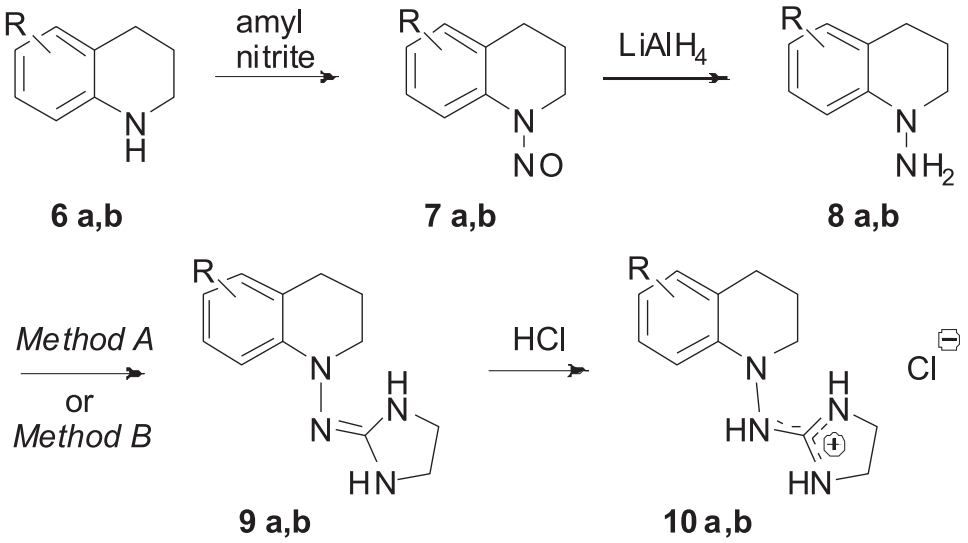
ANOVA was performed for DMAP and DHR, calculated as the difference in MAP and in HR from baseline measurements ("time 0") for each group, as described previously (36). This allowed direct comparisons of responses to treatments between the groups. Data were analyzed with ANOVA with repeated measurements, using Statistica StatSoft



a R = H; **b** R = 2-CH_3; **c** R = 7-CH_3; **d** R = 4-Cl

Method A: 2-chloro-4,5-dihydro-1*H*-imidazole, DCM, 20°C, 12-24 h; for R = H, 2-CH_3
Method B: *N-tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole, acetic acid, 60°C, 16 h; for R = 7-CH_3, 4-Cl

Scheme 1. Synthesis of 1-[(imidazolin-2-yl)amino]indolines



a R = H; **b** R = 8-CH_3;

Method A: 2-chloro-4,5-dihydro-1*H*-imidazole, DCM, 20°C, 12-24 h; for R = H
Method B: *N-tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole, acetic acid, 60°C, 16 h; for R = 8-CH_3

Scheme 2. Synthesis of 1-[(imidazolin-2-yl)amino]-1,2,3,4-tetrahydroquinolines

Table 1. Binding affinities of compounds **5a-d** and **10a**,**b**.

Comp. No.	α_1, K_i (nM)	α_2, K_i (nM)	I_1, IC_{50} (nM)	I_2, K_i (nM)	Selectivity α_1/α_2
5a	1340 ± 7.6	3640 ± 456	123.8 ± 45.4	52.1 ± 25.7	0.368
5b	30.2 ± 2.4	6.09 ± 2.49	39.71 ± 6.05	431.7 ± 276.1	4.96
5c	27.1 ± 4.61	0.75 ± 0.11	1543 ± 1233	48.3 ± 5.93	36.1
5d	106.0 ± 14.07	3.1 ± 0.37	5777 ± 5652	49.8 ± 4.65	34.2
10a	69.8 ± 7.6	4.94 ± 0.66	1423 ± 112.4	14.4 ± 1.36	14,1
10b	528 ± 239	5244 ± 9.19	3076 ± 2647	62.7 ± 5.05	10.1

Table 2. Effect of compounds **5a**, **5c**, **10a** and **10b** at 0.1 mg/kg *i.v.* on mean arterial blood pressure (MAP) in anesthetized rats.

	Time after application of tested compound (min) ΔMAP (mmHg)				
	2	5	15	30	60
5b n = 4	19.9 ± 3.9*	27.1 ± 3.3*	1.9 ± 5.9	-16.0 ± 1.9*	-23.6 ± 1.2*
5c n = 5	25.5 ± 1.7*	29.1 ± 1.8*	-6.3 ± 2.4	-19.0 ± 1.4*	-28.0 ± 1.8*
10a n = 4	23.7 ± 6.8*	37.7 ± 2.8*§	3.4 ± 2.7	-14.5 ± 3.6&	-26.2 ± 2.9*
10b n = 5	21.4 ± 3.8*	14.2 ± 6.7*	-14.4 ± 4.4#$	-14.5 ± 1.9 #	-13.7 ± 2.1 &
Control n = 5	0.0 ± 0.2	0.4 ± 0.7	-0.8 ± 0.7	-1.3 ± 2.0	-3.5 ± 1.8

Values are the mean ± SE. n – number of experiments. Comparisons were made using ANOVA with repeated measures and Fisher test. Significance: (*) $p < 0.001$, (#) $p < 0.002$, (&) $p < 0.05$ *vs.* control group; (§) $p < 0.001$ *vs.* **10b** group; ($) $p < 0.002$ *vs.* **5b** group.

Table 3. Effect of compounds **5b**, **5c**, **10a** and **10b** at 0.1 mg/kg *i.v.* on heart rate (HR) in anesthetized rats.

	Time after application of tested compound (min) ΔHR (bpm)				
	2	5	15	30	60
5b n = 4	-170.4 ± 20.1*&	-131.7 ± 13.9*#	-91.9 ± 22.9*	-88.7 ± 20.0*	-100.6 ± 14.2*#
5c n = 5	-107.3 ± 26.9*#	-94.4 ± 12.8*	-142.3 ± 12.0*#	-154.4 ± 9.2*&	-145.1 ± 13.1*&
10a n = 4	-132.7 ± 24.8*§	-96.0 ± 19.1*	-92.0 ± 16.5*	-96.1 ± 19.7*	-91.7 ± 27.1*
10b n = 5	-61.7 ± 1.4	-79.1 ± 6.9*	-83.0 ± 9.8*	-76.8 ± 5.3*	-58.1 ± 9.3*
Control n = 5	-0.6 ± 1.1	-2.9 ± 0.9	-6.0 ± 1.5	-8.6 ± 2.7	-6.2 ± 6.3

Values are the mean ± SE. n – number of experiments. Comparisons were made using ANOVA with repeated measures and Fisher test. Significance: (*) $p < 0.001$ *vs.* control group; (#) $p < 0.05$, (&) $p < 0.001$, (§) $p < 0.01$ *vs.* **10b** group.

software (StatSoft, Inc., Tulsa, USA). When a treatment effect was significant, *post hoc* comparisons were performed using Fisher's test. A value of $p < 0.05$ was considered statistically significant.

Molecular modeling studies were performed using B3LYP/6-31G* density functional model as implemented into Spartan 08 version 1.2, Wavefunction Inc. Irvine, CA, USA.

RRSULTS AND DISCUSSION

Chemistry

The title indoline-containing (**5a**-**d**) and 1,2,3,4-tetrahydroquinoline-containing (**10a,b**) compounds have been synthesized according to the procedures depicted in Scheme 1 and Scheme 2, respectively. First, the indolines **1** and 1,2,3,4-tetrahydroquinolines **6** were converted into corresponding *N*-nitroso derivatives **2** and **7** by the treatment with amyl nitrite, followed by the reduction with $LiAlH_4$ to give *N*-amino compounds **3** and **8**. Then, upon treatment of **3a**, **3b** and **8a** with 2-chloro-4,5-dihydro-1*H*-imidazole and **3c**, **3d** and **8b** with *N-tert*-butoxycarbonyl-2-methylthio-4,5-dihydro-1*H*-imidazole the desired imidazoline derivatives **4a**-**d** and **9a**,**b** were obtained. For the purposes of biological tests free bases thus obtained were converted into the corresponding hydrochlorides **5a**-**d** and **10a,b**. Structures of the products thus obtained were confirmed by elemental analysis as well as by IR and NMR spectroscopic data.

It is pertinent to note, that the already patented compounds **5a** (23) and **10a** (24) were prepared by different method, i.e., by reacting *N*-amino-indoline **3a** and *N*-amino-1,2,3,4-tetrahydroquinoline **8a**, respectively, with 2-bromoethyl isocyanate followed by imidazoline ring closure upon treatment of corresponding *N*-chloroethylurea with aqueous

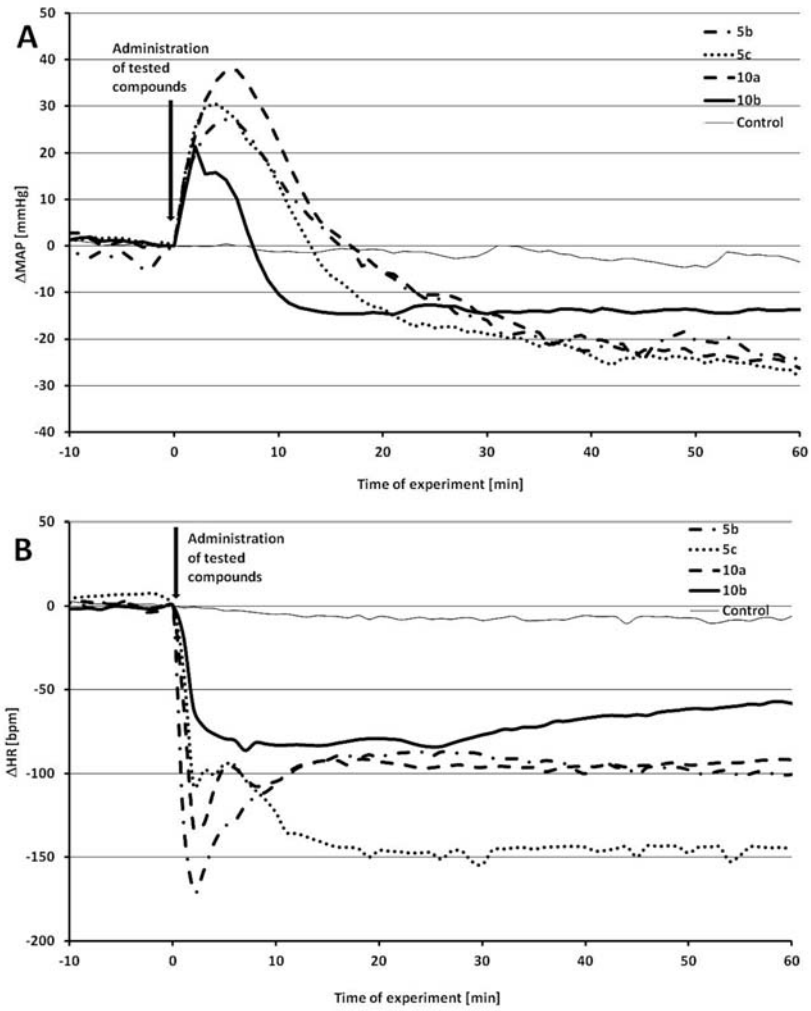


Figure 3. Effect of compounds: **5b**, **5c**, **10a**, **10b**, 100 ěg/kg b.w., and control group on (**A**) ΔMAP and (**B**) ΔHR (calculated as the difference in the MAP or HR between the sequential measurements and time 0 of the experiment) in rats. Each point represents the mean value of ΔMAP or ΔHR for four to five experiments

NaOH. No spectral data for both the free bases or corresponding hydrochloride salts have previously been described.

Binding affinities at α_1-, α_2-adrenoceptors and imidazoline I_1 and I_2 receptors

Radioligand binding experiments of α_2-adrenoceptors and imidazoline I_2 receptors were conducted using crude P_2 rat brain membranes, and crude P_2 rat kidney membranes were used for I_1 receptors. Equilibrium dissociation constants (K_i) were determined by the method of Cheng & Prusoff (35) and the resulting values are presented in Table 1 as the mean ± SEM for 3 or 4 separate experiments.

As shown in Table 1, the unsubstituted compound **5a** showed a poor affinity for both the α_1- and α_2-adrenoceptors with K_i = 1340 nM and 3640 nM, respectively. Compound **5b** with CH_3 substituent at position 2 displayed enhanced activity at α_1- (K_i = 30.2 nM) and α_2- (K_i = 6.09 nM) receptors, but still a negligible α_1/α_2 selectivity ratio of 4.96. The highest difference in potencies at α_1- and α_2-adrenoceptors was showed by 7-CH_3 and 4-Cl -substituted indolines **5c** and **5d** (α_2 K_i = 0.75 and 3.1 nM, respectively; α_1/α_2 selectivity ratio = 36.19 and 34.24, respectively). It should be pointed out that unsubstituted 1,2,3,4-tetrahydroisoquinoline compound **10a** also displayed good affinity for α_2-adrenoceptors (K_i = 4.94 nM) and a moderate affinity for α_1-adrenoceptor (K_i = 69.8 nM), while the 8-CH_3 congener **10b** proved to be less potent.

It is worth mentioning here the noticeable affinities of indoline **5b** at imidazoline I_1 receptors (IC_{50} = 39.7 nM) and 1,2,3,4-tetrahydroquinoline **10a** at imidazoline I_2 receptors (K_i = 14.4 nM).

Effect on arterial blood pressure and heart rate

Intravenous administration of compounds **5b**,**c** and **10a**,**b** at dose 0.1 mg/kg in thiopental-anesthetized male Wistar rats caused a short-lasting pressor response after which significant reduction of arterial blood pressure was observed (Table 2, Fig. 3A). The most pronounced changes in blood pressure were observed for indoline **5c** and 1,2,3,4-tetrahydroquinoline derivative **10a**, i.e., the compounds with relatively high α_1- and α_2-adrenoceptor affinities (Table 1). The initially observed increase in blood pressure resulting from activation of vascular α_1-adrenoceptors (Table 2, Fig. 3A, ΔMAP = 29 and 37 mmHg, respectively) was followed by a long-lasting hypotensive effect (ΔMAP = -28 and -26 mmHg, respectively). Thus, in the circulatory system of Wistar rats the investigated compounds behaved as nonselective stimulators of both α_1- and α_2-adrenoceptors, which was further confirmed by a pronounced bradycardic effect elicited by these compounds (Table 3, Fig. 3B, ΔHR = -154 and -133 bpm, respectively).

The negative chronotropic effect observed in the present study deserves a special attention. It is well known that the human heart expresses α_1-adrenoceptors albeit at much lower levels than β-adrenoceptors (37). However, the role of α_1-receptors in cardiac physiology is still a matter of debate, contrary to their well established effects in regulation of blood flow by inducing constriction of major arteries smooth muscles (38). Very recent study on papillary muscles obtained from rat heart ventricles indicated that stimulation of α_1-adrenoceptors inhibits cardiac excitation-contraction coupling through tyrosine phosphorylation of β_1-adrenoceptors (39). Moreover, experiments performed on human cardiac myocytes indicated expression of α_{1A}- and α_{1B}-adrenoceptors subtypes (40) that are considered as cardioprotective proteins (41). In view of the above information, the immediate sharp fall in the heart rate observed after intravenous administration of tested compounds (Table 3, Fig. 3B) might possibly be mediated by cardiac α_1-adrenoceptors activation.

On the other hand, cardiac function is under the control of the sympathetic and parasympathetic nervous systems. Whereas sympathetic stimulation leads to an increase of cardiac function, the effects of the parasympathetic system are the opposite and vagal stimulation exerts negative inotropic, negative chronotropic and negative chromotropic effects in the heart (42). These observations stay in agreement with our recent experiments performed on vagotomized rats, indicating that although cardiovascular effects of imidazoline compunds of type **B** (Fig. 1, X = NH, *marsanidine*, *7-Me-marsanidine*) are not mediated by the vagal nerves, vagotomy enhanced the sensitivity of sympathetic pathways for tested compounds (43). Therefore, the long-lasting heart rate decrease and hypotensive effect of tested compounds might be mediated through activation of the central α_2-adrenoceptors and the subsequent decrease of sympathetic activity [44, 45].

CONCLUSION

In conclusion, the present studies extend the results previously described in patent literature, showing that imidazoline-containing indolines **5** and 1,2,3,4-tetrahydroquinolines **10** administered at dose of 0.1 mg/kg *i.v.* elicit long-lasting hypotensive and bradycardic effects attributable to their ability to stimulate central α_2-adrenoceptors, and therefore, should

not be classified as hypertensive agents. This study has widened the scope of developing imidazoline derivatives as promising antihypertensive agents.

Acknowledgments

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NEW HETEROCYCLIC OXIME ETHERS OF 1-(BENZOFURAN-2-YL)ETHAN-1-ONE AND THEIR ANTIMICROBIAL ACTIVITY

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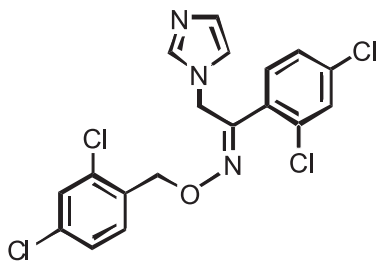
Abstract: In this study, some *O*-benzyl (benzofuran-2-yl)ethan-1-one ether oximes were synthesized starting from 2-acetylbenzofuran. The structure elucidation of the compounds was performed by IR, ^{1}H-NMR and ^{13}C-NMR spectra. Antimicrobial activities of the compounds were examined and notable activity was observed.

Keywords: *O*-benzyl (benzofuran-2-yl)ethan-1-one ether oximes; antimicrobial activity, X-ray analysis

The increase in fungal infections have recently emerged as a growing threat to human health. *Candida* infections are adverse in their appearance (1). The increase in fungal infections and the resistance gained to the currently used drugs in recent years directed the studies on obtaining new antifungal drugs (2). The studies on imidazole and triazole structured antifungal drugs were observed. (3, 4). After discovery of oxiconazole **1**, both azole and ether oximes became of interest. Since then, a number of oximes were synthesized and found to be active against fungi (5-10).



1, oxiconazole

It was proved that the activity of compounds increased when one of the aryl residues was heteroaryl (11). Free oximes and their ethers showed higher activities. When the aryl residue was replaced with benzofuran in a bioisosteric approach, significant antifungal activity was observed (12). In this study, we aimed to obtain compounds derived from oxiconazole, oxime-containing scaffolds. We supposed that if benzofuran is a ring and substituted *O*-benzyl group is in ether oximes, it causes higher activity of such compounds.

Benzofuran is a unique scaffold that is associated with several biological activities. The broad spectrum antifungal (13, 14) and antibacterial activity (15, 16) of these compounds could lead to a new series of antimicrobials. Highly effective compounds were obtained due to aryl benzofuryl ketoximes (17).

EXPERIMENTAL

Chemistry

All reagents were commercially available or synthesized following the procedures described in

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the literature. All NMR spectra were recorded on a Bruker Avance III 700 MHz and Bruker Avance III 400 MHz spectrometer, using $CDCl_3$ as solvent, with TMS as an internal standard. The IR spectra were recorded on Shimadzu FTIR-8400 S spectrometer.

Melting points were determined using an Electrothermal 9100 digital melting point apparatus and were uncorrected.

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone oxime (3)

The suitable 1-(1-benzofuran-2-yl)ethanone (**2**) (3.20 g, 20 mmol), hydroxylamine hydrochloride (1.95 g, 28 mmol) and anhydrous sodium acetate (28 mmol) were refluxed in anhydrous ethanol for 6 h. The reaction mixture was cooled. The crystalline raw product was filtered and recrystallized from anhydrous ethanol (12).

Yield 61%; colorless solid, m.p. 153-154°C, lit. m.p. 155-156°C (18). IR (KBr, cm^{-1}): 3195 (OH). ^{1}H NMR (700 MHz, $CDCl_3$, δ, ppm): 2.37 (s, 3H, CH_3), 2.50 (s, 1H, OH), 7.04 (d, *J* = 0.7 Hz, 1H, CH), 7.28 (dd, *J* = 0.7 Hz, *J* = 7.0 Hz, 1H, CH), 7.37 (ddd, *J* = 0.7 Hz, *J* = 1.4 Hz, *J* = 8.4 Hz, 1H, CH), 7.56 (d, *J* = 8.4 Hz, 1H, CH), 7.62 (dd, *J* = 0.7 Hz, *J* = 8.4 Hz, 1H, CH).

General procedure for preparation of the oxime ethers 4-11 (12)

A mixture of oxime ketone **2** (4 mmol), appropriate benzyl bromide (4 mmol) and potassium carbonate were refluxed in acetone for 8 h. The solvent was evaporated and the residue was washed with water and dried with anhydrous magnesium sulfate. Raw products were crystallized from anhydrous ethanol.

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(4-nitrobenzyl) oxime (4)

Yield 69%, yellow needles, m.p. 127-128°C. IR (KBr, cm^{-1}): 1605 (C=N). ^{1}H NMR (700 MHz, $CDCl_3$, δ, ppm): 2.36 (s, 3H, CH_3), 5.44 (s, 2H, CH_2), 7.05 (d, *J* = 0.7 Hz, 1H, CH), 7.27 (dd, *J* = 0.7 Hz, *J* = 7.0 Hz, 1H, CH), 7.36 (dt, *J* = 1.4 Hz, *J* = 7.0 Hz, 1H, CH), 7.55 (d, *J* = 7.0 Hz, 1H, CH), 7.59 (spin system AA', dt, *J* = 1.4 Hz, *J* = 9.1 Hz, 2H, 2×CH), 7.61 (dd, *J* = 7.7 Hz, 1H, CH), 8.26 (spin system BB', dt, *J* = 2.1 Hz, *J* = 9.1 Hz, 2H, 2×CH). ^{13}C NMR (176 MHz, $CDCl_3$, δ, ppm): 12.35 (CH_3), 75.22 (CH_2), 107.37 (CH), 111.73 (CH), 121.45 (CH), 123.28 (CH), 123.69 (2×CH), 125.85 (CH), 127.82 (C), 128.24 (2×CH), 145.21 (C), 147.57 (C), 148.58 (C), 151.35 (C), 155.25 (C).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(4-chlorobenzyl) oxime (5)

Yield 67%, white needles m.p. 108°C. IR (KBr, cm^{-1}): 1615 (C=N). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.30 (s, 3H, CH_3), 5.30 (s, 2H, CH_2), 7.01 (d, *J* = 0.8 Hz, 1H, CH), 7.25 (dd, *J* = 7.6 Hz, *J* = 0.8 Hz, 1H, CH), 7.35 (dt, *J* = 7.6 Hz, *J* = 1.6 Hz, 1H, CH), 7.36-7.40 (m, 4H, 4×CH), 7.56 (dd, *J* = 0.8 Hz, *J* = 8.4 Hz, 1H, CH), 7.60 (dd, *J* = 0.8 Hz, *J* = 8.4 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.92 (CH_3), 75.88 (CH_2), 104.05 (CH), 112.25 (CH), 121.62 (CH), 122.87 (C), 123.89 (CH), 125.88 (CH), 128.20 (C), 130.05 (2×CH), 131.98 (2×CH), 136.84 (C), 147.52 (C), 151.20 (C), 155.19 (C).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(2,4-dichlorobenzyl) oxime (6)

Yield 70%, white needles m.p. 113-114°C. IR (KBr, cm^{-1}): 1612 (C=N). ^{1}H NMR (700 MHz, $CDCl_3$, δ, ppm): 2.36 (s, 3H, CH_3), 5.42 (s, 2H, CH_2), 7.05 (d, *J* = 0.7 Hz, 1H, CH), 7.27 (dd, *J* = 0.7 Hz, 1H, CH), 7.30 (dd, *J* = 2.1 Hz, *J* = 7.7 Hz, 1H, CH), 7.36 (dt, *J* = 1.4 Hz, 7.0 Hz, 1H, CH), 7.45 (dd, *J* = 2.1 Hz, *J* = 2.8 Hz, 2H, 2×CH), 7.58 (dt, *J* = 0.7 Hz, *J* = 8.4 Hz, 1H, CH), 7.61 (dt, *J* = 0.7 Hz, *J* = 8.4 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.29 (CH_3), 73.27 (CH_2), 107.13 (CH), 111.74 (CH), 121.40 (CH), 123.22 (CH), 125.73 (CH), 127.08 (CH), 127.88 (C), 129.26 (CH), 130.36 (CH), 133.95 (C), 134.15 (C), 148.36 (C), 151.55 (C), 155.25 (C).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(4-bromobenzyl) oxime (7)

Yield 79%, white solid, m.p. 110-111°C. IR (KBr, cm^{-1}): 1616 (C=N). ^{1}H NMR (700 MHz, $CDCl_3$, δ, ppm): 2.36 (s, 3H, CH_3), 5.44 (s, 2H, CH_2), 7.04 (d, *J* = 0.7 Hz, 1H, CH), 7.26 (dd, *J* = 0.7 Hz, *J* = 7.0 Hz, 1H, CH), 7.36 (ddd, *J* = 0.7 Hz, *J* = 1.4 Hz, *J* = 7.0 Hz, 1H, CH), 7.56 (dd, *J* = 0.7 Hz, *J* = 8.4 Hz, 1H, CH), 7.59 (spin system BB`, d, *J* = 9.1 Hz, 2H, 2×CH), 7.61 (d, *J* = 7.7 Hz, 1H, CH), 8.25 (spin system AA', d, *J* = 9.1 Hz, 2H, 2×CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 13.01 (CH_3), 76.69 (CH_2), 101.69 (CH), 112.44 (CH), 122.08 (CH), 122.67 (C), 123.91 (CH), 126.39 (CH), 128.62 (C), 130.57 (2×CH), 132.28 (2×CH), 137.22 (C), 148.66 (C), 152.39 (C), 155.93 (C).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(4-bromo-2-fluorobenzyl) oxime (8)

Yield 72%, white solid, m.p. 88-89°C. IR (KBr, cm^{-1}): 1604 (C=N). ^{1}H NMR (400 MHz,

$CDCl_3$, δ, ppm): 2.30 (s, 3H, CH_3), 5.35 (s, 2H, CH_2), 7.02 (d, *J* = 0.8 Hz, 1H, CH), 7.25 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.30 (dd, *J* = 2.0 Hz, *J* = 4.0 Hz, 1H, CH), 7.33 (d, *J* = 1.6 Hz, 1H, CH), 7.35-7.39 (m, 2H), 7.57 (ddd, *J* = 0.8 Hz, *J* = 2.0 Hz, *J* = 8.8 Hz, 1H, CH), 7.61 (ddd, *J* = 0.8 Hz, *J* = 2.0 Hz, *J* = 8.8 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.22 (CH_3), 69.85 (CH_2), 107.01 (CH), 111.73 (CH), 119.21 (CH), 121.38 (CH), 122.22 (C), 123.20 (CH), 124.01 (C), 125.68 (CH), 127.40 (CH), 127.89 (CH), 131.61 (C), 148.23 (CH), 151.55 (C), 155.25 (C), 159.40 (C), 161.91 (C). ^{19}F NMR (376 MHz, CF_3COOH, δ, ppm): -39.31 (dd, *J* = 7.9 Hz, *J* = 8.3 Hz, C-F, 1F).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(4-trifluoromethylbenzyl) oxime (9)

Yield 75%, white solid, m.p. 82-83°C. IR (KBr, cm^{-1}): 1615 (C=N). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.33 (s, 3H, CH_3), 5.40 (s, 2H, CH_2), 7.03 (d, *J* = 1.2 Hz, 1H, CH), 7.26 (dd, *J* = 7.6 Hz, *J* = 0.8 Hz, 1H, CH), 7.35 (dt, *J* = 7.6 Hz, *J* = 1.2 Hz, 1H, CH), 7.54-7.57 (m, 2H, 2×CH), 7.60 (ddd, *J* = 0.8 Hz, *J* = 1.2 Hz, *J* = 7.6 Hz, 1H, CH), 7.67 (d, *J* = 7.6 Hz, 1H, CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.30 (CH_3), 75.81 (CH_2), 107.11 (CH), 111.73 (CH), 121.39 (CH), 123.22 (CH), 125.33 (CH), 125.37 (C), 125.41 (C), 125.73 (CH), 127.86 (CH), 128.06 (CH), 141.63 (C), 148.20 (C), 148.58 (C), 151.57 (C), 155.24 (C). ^{19}F NMR (376 MHz, CF_3COOH, δ, ppm): 13.48 (s, 3F, CF_3).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(2-fluorobenzyl) oxime (10)

Yield 64%, white-yellow solid, m.p. 58-59°C. IR (KBr. cm^{-1}): 1618 (C=N). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.31 (s, 3H, CH_3), 5.43 (s, 2H, CH_2), 7.02 (d, *J* = 0.8 Hz, 1H, CH), 7.10 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.18 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.25 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.30-7.37 (m, 2H, 2×CH), 7.51 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.55-7.61 (m, 2H, 2×CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.22 (CH_3), 70.50 (CH_2), 106.84 (CH), 111.73 (CH), 115.46 (C), 123.16 (CH), 124.08 (CH), 124.67 (C), 125.60 (CH), 127.95 (CH), 129.82 (CH), 130.56 (CH), 147.95 (C), 151.80 (C), 155.23 (C), 159.72 (C), 162.19 (C). ^{19}F NMR (376 MHz, $CDCl_3$, δ, ppm): -42.17 (ddd, *J* = 6.8 Hz, *J* = 7.5 Hz, *J* = 8.3 Hz, C-F, 1F).

(*E*)-1-(benzo[*b*]furan-2-yl)ethanone *O*-(2,6-difluorobenzyl) oxime (11)

Yield 69%, white needles, m.p. 73-75°C. IR (KBr, cm^{-1}): 1626 (C=N). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 2.25 (s, 3H, CH_3), 5.43 (s, 2H, CH_2), 6.95 (dd, *J* = 1.2 Hz, *J* = 7.2 Hz, 2H, 2×CH), 7.00 (d, *J* = 0.8 Hz, 1H, CH), 7.25 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 1H, CH), 7.31-7.37 (m, 2H, 2×CH), 7.58 (dd, *J* = 0.8 Hz, *J* = 7.2 Hz, 2H, 2×CH). ^{13}C NMR (100 MHz, $CDCl_3$, δ, ppm): 12.04 (CH_3), 64.11 (CH_2), 106.71 (CH), 111.18 (C), 111.43 (C), 111.73 (CH), 112.92 (C), 121.34 (CH), 123.13 (CH), 125.54 (CH), 127.97 (CH), 130.54 (CH), 148.03 (CH), 151.80 (C), 155.21 (C), 160.99 (C), 163.49 (C). ^{19}F NMR (376 MHz, $CDCl_3$, δ, ppm): -38.18 (t, *J* = 6.8 Hz, 2F, 2×C-F)

Crystal structure determination of 10

Crystal data: $C_{17}H_{14}FNO_2$, M_r = 283.29, monoclinic, space group $P2_1/n$, *a* = 10.2639(3), *b* = 5.7270(2), *c* = 23.9296(7) Å, β = 94.132(3)§, *V* = 1402.97(7) Å^3, *T* = 293(2) K, *Z* = 4 (*Z*' = 1).

Data collection: A colorless block crystal (ethanol) of 0.40 × 0.22 × 0.13 mm was used to record 16216 (Mo*K*α radiation, θ_{max} = 29.09°) intensities on an Agilent Xcalibur A diffractometer. Intensity data collection employed the ω- scans mode with "Enhance (Mo) X-ray Source". The data were corrected for Lorentz and polarization effects. Data reduction and analysis were carried out with the CrysAlis PRO program (19). The 3506 total unique reflections (R(int) = 0.023) were used for further calculations.

Structure solution and refinement: The structure was solved by the direct methods using the program SHELXS-97 (20) and refinement was done against F^2 for all data using SHELXL-97 (20). The positions of the H atoms were positioned geometrically and were refined using a riding model, with C–H = 0.96 Å (CH_3), 0.97 Å (CH_2), 0.93 Å ($C_{sp}2H$) and U_{iso}(H) = 1.2U_{eq}(C) or 1.5U_{eq}(C) for methyl H atoms. The methyl group was refined as a rigid group, which was allowed to rotate. The final refinement converged with *R* = 0.0492 (for 2399 data with $I > 2\sigma(I)$), *wR* = 0.1369 (on F^2 for all data), and *S* = 1.048 (on F^2 for all data). The largest difference peak and hole were 0.294 and -0.218 eÅ^{-3}. The molecular illustration was drawn using ORTEP-3 for Windows (21). Software used to prepare material for publication was WINGX (21) and PLATON (22).

The supplementary crystallographic data have been deposited at the Cambridge Crystallographic Data Centre (CCDC), 12 Union Road, Cambridge CB2 1EZ (UK), phone: (+44) 1223/336 408, fax: (+44) 1223/336 033, e-mail: deposit@ccdc.cam.ac.uk, World Wide. Web:http://www.ccdc.cam.ac.uk (deposition No. CCDC 975847).

Antibacterial and antifungal activity

The antimicrobial activities of the compounds were determined using the broth microdilution reference method (23) in standard 96-well polystyrene plates (Kartell). The tested microorganisms were Gram-negative *Escherichia coli* ATTC 25922 and Gram-positive *Staphylococcus aureus* ATCC 25923 bacteria, the yeasts *Candida albicans* and *Malessezia pachydermatis* CBS7925. The study was carried out using microdilution method with the following dilutions of the tested compounds from 400 to 6.25 μg/mL for *Malessezia* (24) and from 512 to 0.125 μg/mL for bacteria and *Candida*.

The bacterial strains were cultivated in Luria-Bertani (LB) broth and the yeast in Sabouraud dextrose broth (SDB). The tested compounds were dissolved in dimethyl sulfoxide (DMSO); diluted tenfold with culture broth to a concentration of 1.024 mg/mL, and then serially diluted in the appropriate medium. The wells were inoculated with the tested strains to a final concentration of 10^4 CFU/mL. The control sample included inoculated growth medium without the compound. In all the tests, DMSO was used as the control; DMSO had no effect on the microorganisms in the concentrations studied (up to 2.5%). Ampicillin (Polfa Tarchomin SA) and itraconazole (Janssen-Cilag International NV) were used as antibiotic reference for the bacteria and yeast, respectively. The plates were incubated at 37°C for 24 h for the bacteria, 48 h for *Candida* and 72 h for *Malessezia*. The microbial growth rate was measured as an optical density at 550 nm (OD_{550}). The tests were performed in triplicate for each concentration.

The minimum inhibitory concentrations (MIC) were defined as the lowest concentration of the compounds at which no visible growth of the tested microorganism occurred.

RESULTS AND DISCUSSION

Chemistry

The final products (**4**-**11**) were synthesized as outlined in Scheme 1. Ketone **2** was prepared from salicylic aldehyde with chloroacetone (25). The (*E*)-oxime **3** was prepared from ketone **2** and recrystallized from ethanol.

Oxime **3** was reacted with appropriate substituted benzyl bromides with high yields. The oxime ethers can be prepared in a reaction with oxime and sodium (26) or sodium hydride (27).

As expected, the presence of *E* and *Z* isomers of the oxime derivatives was observed in the raw products. All the final products were crystallizes from ethanol. Thus, no isomers *Z* were observed in the final products; in the NMR spectra aliphatic pro-

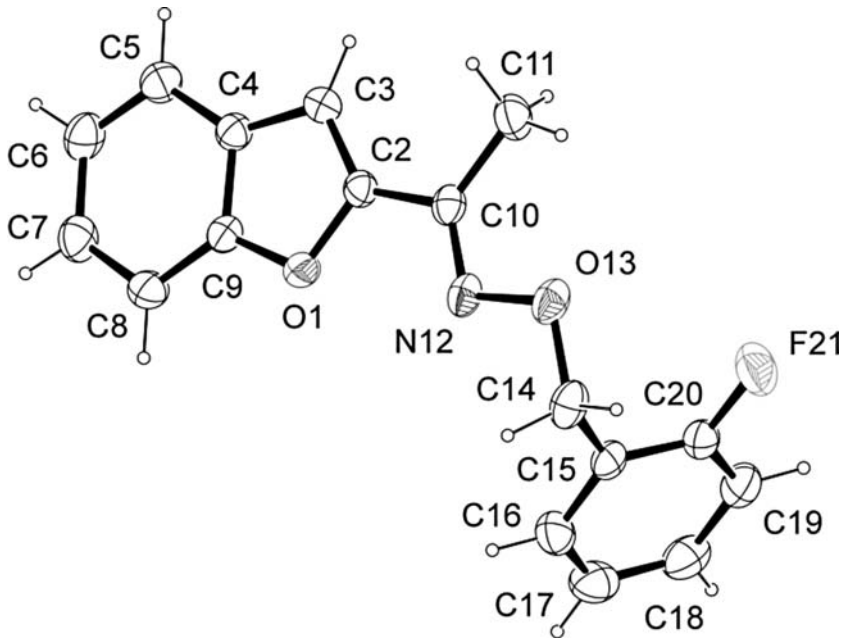
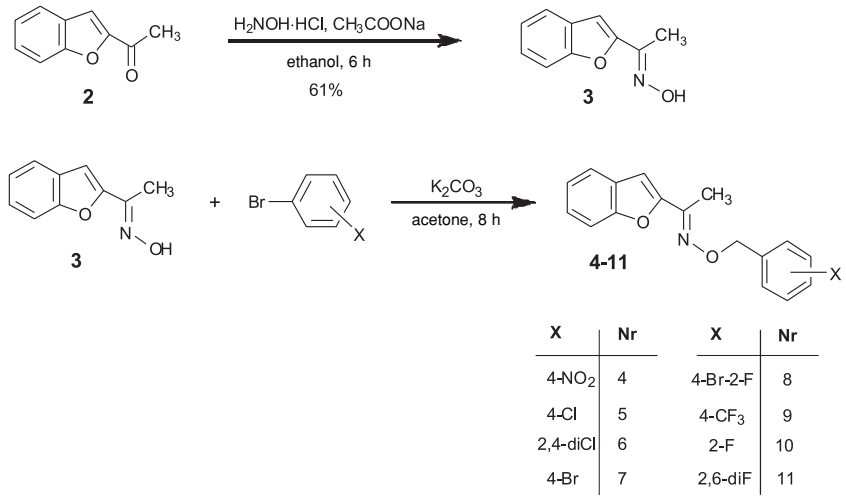


Figure 1. The molecular structure of compound **10** showing the atom labelling scheme. Non-H atoms are drawn as 30% probability displacement ellipsoids and H atoms are shown as small spheres of arbitrary radius



X	Nr	X	Nr
4-NO_2	4	4-Br-2-F	8
4-Cl	5	4-CF_3	9
2,4-diCl	6	2-F	10
4-Br	7	2,6-diF	11

Scheme 1. Reaction sequence for the synthesis of ether oximes **4**-**11**

Table 1. Antibacterial and antifungal activities of the compounds **4**-**11** and the standard drugs used in the study (MIC μg/mL).

Compound	MIC [μg/mL]		
	Staphylococcus aureus	*Escherichia coli*	*Candida albicans*
4	> 512	512	512
5	> 512	512	512
6	256	> 512	512
7	256	512	512
8	256	256	512
9	128	512	512
10	256	512	256
11	256	512	256
Itraconazole	-	-	2
Ampicillin	8	8	-

tons were not resonated in two different groups with corresponding integral values. We have prepared a few fluorinated products **8**-**11**, supposedly being highly effective. Structural features of synthesized heterocyclic oxime ethers of 1-(benzofuran-2-yl)ethan-1-one were confirmed by X-ray crystallographic analysis of exemplified compound **10**.

Crystallographic data

The molecular structure of compound **10** and the atom-labelling scheme is illustrated in Figure 1.

The nine-membered benzofuran system is planar with an r.m.s. deviation of 0.0083 Å and is in *E* configuration with respect to the 2-fluorobenzyloxy moiety [torsion angle C2-C10-N12-O13: -178.87 (11)°]. Simultaneously, conjugated system of double bonds C2 = C3 and C10 = N12 has *s-trans* conformation [torsion angle C3-C2-C10-N12: 179.62 (16)°].

The interatomic distance C10 = N12 takes the value of 1.286(2) Å and confirms the occurrence of the double bond between these atoms.

Angular orientation of the 2-fluorobenzyloxy fragment in the molecule reveal three torsional angles C10-N12-O13-C14, N12-O13-C14-C15 and O13-C14-C15-C16 of -174.93(13), 74.23(16) and

66.49(19)°, respectively. The first one indicates that the C10-N12 and O13-C14 bonds are antiperiplanar to each other while the second and the third torsional angles both reveal mutual synclinal orientation of the bonds N12-O13 and C14-C15 or O13-C14 and C15-C16. The phenyl ring of the 2-fluorobenzyloxy moiety forms a dihedral angle of 80.03(5)° with the planar benzofuran system.

Antimicrobial activity

The antimicrobial activity of the synthesized compounds (**4**-**11**) was evaluated *in vitro* against *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *C. albicans,* and *M. pachydermatis*. The results of the evaluation of their minimal inhibitory concentration values are presented in Table 1. Two standard drugs ampicillin and itraconazole were used as controls; the MIC values obtained for these compounds are 8 and 2 μg/mL, respectively.

The growth of Gram-positive reference strain *S. aureus* was inhibited by compounds **6**, **7**, **8**, **10** and **11** at the concentration of 250 μg/mL. A twofold lower dose (128 μg/mL) was required in the case of derivative **9,** carrying a 4-trifluoromethylbenzyl substituent, to inhibit the growth of *S. aureus*. Of all the tested substances, only compound **8** (4-bromo-2-fluorobenzyl group) was active against *E. coli* (MIC 256 μg/mL). Derivatives **10** and **11**, with 2-fluorine and 2,6-difluorine groups, showed moderate activity against *C. albicans*, MIC values of 250 mg/mL, but were less active than the standard antifungal drug, itraconazole.

The other derivatives examined, i.e., oxime ethers **4** and **5**, were inactive against all the tested microorganisms (MIC ≥ 512 μg/mL).

Evaluation of the antifungal activity of the tested oxime ethers against *M. pachydermatis* showed a slight inhibitory effect at the concentration of 400 μg/mL. The growth of the tested *Malessezia* strains was half less intense as against the positive control - an inoculum of the fungus in Sabouraud medium without the compounds. All the tested derivatives were ineffective at lower concentrations (200-6.25 μg/mL).

Alper-Hayta et al. (16) showed that 2-(substituted phenyl/benzyl)-5-[(2-benzofuryl)carboxamido]-benzoxazole derivatives possessed a broad spectrum of activity against Gram-positive and Gram-negative bacteria as well as *Candida* (MIC range between 15.625-500 μg/mL). Similar results were obtained by other authors; the compounds of cyclobutane substituted benzofuran class were able to inhibit the growth of *C. albicans* and *S. aureus* at the concentration from 2.5 to 0.039 mg/mL (15). The dinaphtho[2,1-*b*]furan-2-yl-methanone compounds and their oxime derivatives showed weak antimicrobial activity against bacteria and *Candida* (128-512 μg/mL) (28).

We have described the synthesis and antibacterial and antifungal activity of new benzofuran-containing oximes. The results obtained showed that these compounds exhibited relatively weak antimicrobial potency against the tested microorganisms. The most promising activity was detected in derivatives **8**–**11** containing fluorine residues.

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Conflicts of interests

The Authors have declared that there is no conflict of interests.

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SYNTHESIS AND *IN VITRO* ANTIPROLIFERATIVE SCREENING OF NEW 2,7-NAPHTHYRIDINE-3-CARBOXYLIC ACID HYDRAZIDE DERIVATIVES

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Abstract: The new pyrrolo[3,4-*c*]pyridines and 2,7-naphthyridine derivatives have been synthesized. 4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide (**4**) was the key intermediate for the synthesis of the novel derivatives of various chemical structures: Schiff bases, 1,3,4-oxadiazoles, pyrazoles, carbohydrazides, semi- and thiosemicarbazides. The structures of these new compounds were confirmed by elemental analysis and IR, NMR and MS spectra. The antitumor activities of the obtained derivatives were examined. Eight of the twenty one newly synthesized compounds were qualified by the NCI (Bethesda, MD, USA) for *in vitro* screening against 60 different human tumor cell lines. The most active proved to be the Schiff bases.

Keywords: pyrrolo[3,4-*c*]pyridine-1,3-dione, 2,7-naphthyridine derivatives, Schiff bases, antiproliferative activity *in vitro*

2,7-Naphthyridine is one of the six structural isomers of pyridopyridine. The reviews (1, 2) showed that natural alkaloids and synthetic compounds, containing the 2,7-naphthyridine scaffold, exhibit a broad spectrum of biological activities. Most of them have been studied as antitumor agents (3-7). Antibacterial (3, 8, 9), antifungal (10, 11), anti-inflammatory (12), antimalarial (13, 14), analgesic, and anticonvulsant (15, 16) activities were also examined. The various biological properties of 2,7-naphthyridines encourage the search for new methods of their preparation.

In our previous paper (17), a way of synthesizing 2,7-naphthyridine ring has been determined by alkoxide-induced rearrangement of pyrrolo[3,4-*c*]pyridines. The structure of new compounds was determined by X-ray crystallography to prove the presence of 2,7-naphthyridine isomer (17). Most of the newly synthesized 6-phenyl-2,7-naphthyridine derivatives were evaluated against the different human tumor cell lines, representing leukemia, melanoma, and CNS, breast, colon, kidney, ovary, prostate, and non-small cell lung cancers. In our studies, we have found that the most active compounds were the 4-hydroxy-1-oxo-6-phenyl-2,7-naphthyridine-3-carboxylic acid hydrazide derivatives (GI_{50} values between 0.24–3.48 μmol) (18). The present work is a follow-up study to our recent articles (17, 18).

The aim of this paper was to synthesize the new 4-methyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-diones **2a**-**c** and 8-methyl-4-hydroxy-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylate derivatives **3a**-**b**, **5**-**13**, according to the method reported earlier (17–20). Selected compounds were tested for their antiproliferative activity *in vitro*.

EXPERIMENTAL

Chemistry

Melting points were measured in open glass capillaries with a MEL-TEMP apparatus (Barnstead International, Dubuque, IO, USA) and were uncorrected. The new products were analyzed using a Perkin Elmer 2400 analyzer (Waltham, MA, USA). IR spectra were performed on a Specord M80 spectrometer (Zeiss/Analytic Jena, Germany) using KBr pellets. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 with a Bruker Avance ARX-300 MHz spectrometer (Bruker Analytic, Karlsruhe, Germany) with TMS as the internal standard. MS spectra were determined on a GCMS-LK82091 spectrometer at the ionization energy 70 eV. The course of the reactions and the purity of the com-

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pounds were checked by TLC using aluminum sheet silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany). The chemicals for the syntheses were purchased from Chempur, Alfa Aesar, and Lancaster. Compounds **1** and **4** were prepared according to the methods presented in our previous papers (17, 18).

General procedure for the synthesis of 4-methyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-dione derivatives (2a-c)

To a solution of 4-methyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-dione **1** (0.01 mol) in anhydrous *N*,*N*-dimethylformamide (100 mL), sodium hydride (0.01 mol) was added. The mixture was stirred at room temperature for 2 h. To obtained sodium salt, methyl bromoacetate (0.01 mol), or benzyl bromoacetate (0.01 mol), or 2-bromoacetophenone (0.01 mol) was dropped. The mixture was stirred at room temperature for 4-6 h, and next, it was diluted with water. The obtained solid was filtered, dried and crystallized.

Methyl 2-(4-methyl-1,3-dioxo-6-phenyl-pyrrolo[3,4-*c*]pyridin-2-yl)acetate (2a)

Yield 2.82 g (91%), yellow solid, crystallized from methanol, m.p. 152-154°C. IR (KBr, cm^{-1}): 1780, 1740, 1720 (C=O), 1260 (CO), 750 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.82 (s, 3H, CH_3), 3.71 (s, 3H, OCH_3), 4.46 (s, 2H, CH_2), 7.52 (m, 3H, phenyl), 8.26 (m, 3H, pyridine, phenyl). MS (70 eV): m/z (%) : 311 [(M + 1)+, 5], 310 (M^+, 37), 252 (12), 251 (100), 250 (3), 195 (2), 154 (5), 153 (3), 127 (6), 126 (4), 104 (2), 77 (4), 59 (2). Analysis: calcd. for $C_{17}H_{14}N_2O_4$ (310.31): C, 65.80; H, 4.55; N, 9.03%; found: C, 65.71; H, 4.61; N, 9.15%.

Benzyl 2-(4-methyl-1,3-dioxo-6-phenyl-pyrrolo[3,4-*c*]pyridin-2-yl)acetate (2b)

Yield 3.24 g (84%), white solid, crystallized from ethanol, m.p. 182-184°C. IR (KBr, cm^{-1}): 1740, 1720, 1620 (C=O), 1220 (CO), 750, 700 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.85 (s, 3H, CH_3), 4.53 (s, 2H, CH_2), 5.20 (s, 2H, CH_2), 7.37 (m, 5H, phenyl), 7.54 (m, 3H, phenyl), 8.27 (m, 2H, phenyl), 8.32 (s, 1H, pyridine). Analysis: calcd. for $C_{23}H_{18}N_2O_4$ (386.41): C, 71.49; H, 4.70; N, 7.25%; found: C, 71.11; H, 4.58; N, 7.22%.

4-Methyl-2-phenacyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-dione (2c)

Yield 2.56 g (72%), white solid, crystallized from ethanol, m.p. 217-218°C. IR (KBr, cm^{-1}): 1710, 1700 (C=O), 750, 680 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.86 (s, 3H, CH_3), 5.26 (s, 2H, CH_2), 7.54 (m, 3H, phenyl), 7.60 (m, 2H, phenyl), 7.74-8.10 (m, 3H, phenyl), 8.28 (m, 2H, phenyl), 8.31 (s, 1H, pyridine). Analysis: calcd. for $C_{22}H_{16}N_2O_3$ (356.38): C, 74.15; H, 4.53; N, 7.86%; found: C, 74.12; H, 4.62; N, 8.11%.

General procedure for the synthesis of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine derivatives (3a,b)

To a solution of sodium ethoxide (0.04 mol) in anhydrous ethanol the appropriate 4-methyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-dione derivatives **2a-c** (0.01 mol) were added. The mixture was heated at 60°C with stirring for 1 h. After cooling, the mixture was diluted with ice-water and acidified with 10% hydrogen chloride to pH = 5-6. The obtained solid was filtered, dried and crystallized.

Ethyl 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylate (3a)

Yield: 2.11 g (65%), beige solid, crystallized from methanol, m.p. 232-234°C (lit. 233-235°C) (17). IR (KBr, cm^{-1}): 3440 (NH), 2900 (OH), 1650 (C=O), 1280 (C-O), 770 (CH, arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.38 (t, J = 7.0 Hz, 3H, CH_3), 3.05 (s, 3H, CH_3), 4.41 (q, J = 8.9 Hz, 2H, CH_2), 7.54 (m, 3H, phenyl), 8.18 (m, 3H, phenyl, pyridine), 8.41 (s, 1H, OH), 10.52 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ, ppm): = 13.8, 26.4, 62.3, 109.5, 112.8, 119.5, 127.0 (2C), 128.9 (2C), 129.9, 137.5, 140.2, 141.7, 156.5, 158.5, 161.7, 163.2. Analysis: calcd. for $C_{18}H_{16}N_2O_4$ (324.34): C, 66.66; H, 4.97; N, 8.64%; found: C, 66.81; H, 4.80; N, 8.69%.

3-Benzoyl-4-hydroxy-8-methyl-6-phenyl-2*H*-2,7-naphthyridin-1-one (3b)

Yield 2.49 g (70%), yellow solid, crystallized from ethanol, m.p. 252-255°C. IR (KBr, cm^{-1}): 3450 (NH), 1650, 1620 (C=O), 1280 (CO), 780, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.12 (s, 3H, CH_3), 7.55 (m, 4H, phenyl), 7.66 (m, 2H, phenyl), 7.91 (m, 2H, phenyl), 8.21 (m, 3H, phenyl, pyridine), 8.41 (s, 1H, OH), 11.10 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ, ppm): 27.3, 112.5, 118.1, 121.9, 124.5, 126.8 (2C), 127.1, 128.3 (2C), 128.5, 128.9 (2C), 129.8 (2C), 130.2, 132.5, 136.9, 137.1, 138.2, 154.3, 159.1. Analysis: calcd. for $C_{22}H_{16}N_2O_3$ (356.38): C, 74.15; H, 4.53; N, 7.86%; found: C, 73.96; H, 4.45; N, 7.45%.

General procedure for the synthesis of Schiff bases (5a-i)

To a solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in ethanol (100 mL) the appropriate aldehyde (0.01 mol) and catalytic amount of indium (III) trifluoromethanesulfonate were added. The mixture was refluxed with stirring for 2-4 h. After cooling, the precipitate was filtered off. Recrystallization from the proper solvents afforded the Schiff bases **5a-i**.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid phenethylidene-hydrazide (5a)

Yield 1.69 g (41%), yellow solid, crystallized from toluene, m.p. 270-272°C. IR (KBr, cm^{-1}): 3350, 3200 (NH), 3000 (CH), 1650 (C=O), 1580 (C=N), 1280 (CO), 770, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, CH_3), 3.33 (d, J = 7.0 Hz, 2H, CH_2), 7.51-7.59 (m, 5H, phenyl), 8.01-8.17 (m, 5H, phenyl), 8.42 (s, 1H, CH), 8.86 (s, 1H, pyridine), 9.37 (s, 1H, OH), 10.68 (br, 1H, NH), 12.21 (s, 1H, NH). Analysis: calcd. for $C_{24}H_{20}N_4O_3$ (412.44): C, 69.89; H, 4.89; N, 13.38%; found: C, 69.98; H, 4.59; N, 13.26%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid propylidene-hydrazide (5b)

Yield 1.57 g (45%), yellow solid, crystallized from ethanol, m.p. 284-285°C. IR (KBr, cm^{-1}): 3430, 3240 (NH), 2950 (CH), 1650 (C=O), 1580 (C=N), 1350 (CO), 750, 700 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 0.97 (t, J = 6.4 Hz, 3H, CH_3), 1.63-1.78 (q, J = 6.8 Hz, 2H, CH_2), 3.07 (s, 3H, CH_3), 5.45 (t, J = 8.7 Hz, 1H, CH), 6.35 (s, 1H, phenyl), 7.47-7.56 (m, 3H, phenyl), 8.17-8.21 (m, 3H, phenyl, pyridine, OH), 10.14 (s, 1H, NH), 12.34 (s, 1H, NH). Analysis: calcd. for $C_{19}H_{18}N_4O_3$ (350.37): C, 65.13; H, 5.18; N, 15.99%; found: C, 65.08; H, 5.28; N, 15.79%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2-hydroxybenzylidene)-hydrazide (5c)

Yield 1.60 g (39%), yellow solid, crystallized from toluene, m.p. 272-273°C. IR (KBr, cm^{-1}): 3330, 3000 (NH), 1630 (C=O), 1580 (C=N), 1360, 1270 (CO), 750, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.07 (s, 3H, CH_3), 6.95 (m, 2H, phenyl), 7.33 (s, 1H, CH), 7.53-7.66 (m, 5H, phenyl), 8.20-8.24 (m, 3H, phenyl, pyridine), 8.40 (s, 1H, OH), 8.63 (s, 1H, OH), 10.91 (s, 1H, NH), 12.17 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{18}N_4O_3$ (414.42): C, 66.69; H, 4.38; N, 13.50%; found: C, 66.31; H, 3.98; N, 13.88%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2-chlorobenzylidene)-hydrazide (5d)

Yield 1.50 g (35%), yellow solid, crystallized from toluene, m.p. 286-288°C. IR (KBr, cm^{-1}): 3350 (NH), 2950 (CH), 1640 (C=O), 1580 (C=N), 1360 (CO), 750, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.06 (s, 3H, CH_3), 7.51-7.59 (m, 5H, phenyl), 8.00 (s, 1H, CH), 8.17-8.20 (m, 4H, phenyl), 8.71 (s, 1H, pyridine), 10.65 (s, 1H, OH), 11.29 (s, 1H, NH), 12.23 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{17}ClN_4O_3$ (432.86): C, 63.82; H, 3.96; N, 12.94%; found: C, 64.21; H, 3.89; N, 12.65%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (4-fluorobenzylidene)-hydrazide (5e)

Yield 1.99 g (48%), yellow solid, crystallized from toluene, m.p. 312-314°C. IR (KBr, cm^{-1}): 3330 (NH), 2930 (CH), 1650 (C=O), 1580 (C=N), 1240 (CO), 780, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.09 (s, 3H, CH_3), 7.22-7.33 (m, 4H, phenyl), 7.54-7.56 (m, 2H, phenyl), 7.71-7.80 (m, 4H, phenyl, pyridine), 8.13 (s, 1H, CH), 8.59 (s, 1H, OH), 8.69 (s, 1H, NH), 12.19 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{17}FN_4O_3$ (416.40): C, 66.34; H, 4.11; N, 13.45%; found: C, 66.27; H, 3.90; N, 13.64%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (3-phenylallylidene)-hydrazide (5f)

Yield 2.41 g (57%), yellow solid, crystallized from toluene, m.p. 232-234°C. IR (KBr, cm^{-1}): 3340 (NH), 2950 (CH), 1640 (C=O), 1590 (C=N), 1240 (CO), 750, 700 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.07 (s, 3H, CH_3), 7.10-7.21 (m, 2H, CH), 7.34-7.40 (m, 3H, phenyl), 7.49-7.55 (m, 3H, phenyl), 7.63-7.65 (m, 2H, phenyl), 8.14-8.22 (m, 4H, phenyl, pyridine, CH), 10.61 (s, 1H, OH), 11.90 (s, 1H, NH), 12.22 (s, 1H, NH). Analysis: calcd. for $C_{25}H_{20}N_4O_3$ (424.45): C, 70.74; H, 4.75; N, 13.20%; found: C, 70.69; H, 4.51; N, 13.41%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2,4-dihydroxybenzylidene)-hydrazide (5g)

Yield 2.58 g (60%), beige solid, crystallized from toluene, m.p. 286-288°C. IR (KBr, cm^{-1}): 3380 (NH), 1630 (C=O), 1580 (C=N), 1360, 1340, 1230 (CO), 770, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.06 (s, 3H, CH_3), 6.29-6.33 (m, 2H, OH), 7.38-7.41 (d, J = 8.1 Hz, 1H, CH), 7.51-7.60 (m, 4H, phenyl), 8.17-8.23 (m, 4H, phenyl), 8.46 (s, 1H, pyridine), 10.05 (s, 1H, OH), 11.18 (s, 1H, NH),

12.20 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{18}N_4O_5$ (430.41): C, 64.18; H, 4.22; N, 13.02%; found: C, 64.43; H, 3.95; N, 13.14%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2,4,6-trimethoxybenzylidene)-hydrazide (5h)

Yield 2.04 g (42%), yellow solid, crystallized from toluene, m.p. 280-282°C. IR (KBr, cm^{-1}): 3340 (NH), 2930 (CH), 1640 (C=O), 1580 (C=N), 1360, 1320, 1230, 1120 (CO), 780, 700 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.04 (s, 3H, CH_3), 3.69 (s, 3H, CH_3), 3.82 (s, 6H, CH_3), 6.94-6.97 (m, 2H, phenyl, CH), 7.51-7.58 (m, 3H, phenyl), 8.18-8.26 (m, 4H, phenyl, pyridine), 10.68 (s, 1H, OH), 12.00 (s, 1H, NH), 12.53 (s, 1H, NH). Analysis: calcd. for $C_{26}H_{24}N_4O_6$ (488.49): C, 63.90; H, 5.00; N, 11.50%; found: C, 64.27; H, 4.65; N, 11.32%.

4-Hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (3-nitrophenylbenzylidene)-hydrazide (5i)

Yield 3.01 g (68%), orange solid, crystallized from toluene, m.p. 284-285°C. IR (KBr, cm^{-1}): 3350 (NH), 2930 (CH), 1660 (C=O), 1580 (C=N), 1540, 1360 (NO), 1250 (CO), 740, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.03 (s, 3H, CH_3), 7.16 (s, 1H, CH), 7.50-7.71 (m, 4H, phenyl), 8.14-8.52 (m, 5H, phenyl), 8.71 (s, 1H, pyridine), 10.11 (br, 2H, NH, OH), 12.61 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{17}N_5O_5$ (443.41): C, 62.41; H, 4.58; N, 16.35%; found: C, 62.69; H, 4.45; N, 15.98%.

4-Hydroxy-8-methyl-6-phenyl-3-(5-phenyl-1,3,4-oxadiazol-2-yl)-2*H*-2,7-naphthyridin-1-one (6)

To the solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in anhydrous DMF (30 mL), benzoic acid (0.01 mol) and phosphorous oxychloride (3 mL) were added. The mixture was refluxed with stirring for 3 h. After cooling, the mixture was diluted with ice-water and then neutralized with sodium bicarbonate. The obtained solid was filtered, dried and crystallized.

Yield 1.62 g (41%), beige solid, crystallized from ethanol, m.p. 296-297°C. IR (KBr, cm^{-1}): 3400 (NH, OH), 2930 (CH), 1660, 1580 (C=N, C=O), 1190 (CO), 790, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, CH_3), 7.51-7.54 (m, 6H, phenyl, pyridine), 8.03-8.20 (m, 5H, phenyl), 11.60 (br, 1H, OH), 12.93 (s, 1H, NH). Analysis: calcd. for $C_{23}H_{16}N_4O_3$ (396.40): C, 69.78; H, 4.10; N, 14.11%; found: C, 70.16; H, 4.08; N, 14.50%.

4-Hydroxy-8-methyl-3-(1,3,4-oxadiazol-2-yl)-6-phenyl-2*H*-2,7-naphthyridin-1-one (7)

The mixture of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) and triethyl orthoformate (0.01 mol) in ethanol (50 mL) was refluxed with stirring for 5 h. After cooling, the obtained solid was filtered, dried and crystallized.

Yield 2.02 g (63%), yellow solid, crystallized from ethanol, m.p. 300-302°C. IR (KBr, cm^{-1}): 3450, 3200, 2900 (OH, NH), 1650 (C=O), 1580 (C=N), 1300 (CO), 760 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 2.99 (s, 3H, CH_3), 7.50-7.55 (m, 3H, phenyl), 8.02-8.29 (m, 4H, phenyl, pyridine, oxadiazole), 11.55 (s, 1H, OH), 12.12 (s, 1H, NH). ^{13}C NMR (DMSO-d_6, δ, ppm): 26.96, 108.43, 110.32, 117.31, 127.07 (2C), 128.07, 128.88 (2C), 130.35, 136.78, 138.01, 139.46, 154.79, 156.74, 160.19, 162.52. Analysis: calcd. for $C_{17}H_{12}N_4O_3$ (320.30): C, 63.70; H, 3.80; N, 17.51%; found: C, 63.86; H, 4.05; N, 17.11%.

5-(4-Hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridin-3-yl)-3*H*-1,3,4-oxadiazol-2-one (8)

To solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in anhydrous tetrahydrofuran (50 mL) 1,1-carbonyldiimidazole (0.02 mol) was added. The mixture was stirred for 18 h. The solvent was evaporated under reduced pressure. The precipitate was washed with ethanol and filtered, dried and crystallized.

Yield 1.31 g (38%), beige solid, crystallized from ethanol, m.p. 327-330°C. IR (KBr, cm^{-1}): 3100 (OH), 2900 (NH), 1650, 1600 (C=O), 1500 (CN), 820 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.02 (s, 3H, CH_3), 7.48-7.50 (m, 3H, phenyl), 8.08-8.15 (m, 3H, phenyl, pyridine), 11.61 (br, 3H, NH, OH). Analysis: calcd. for $C_{17}H_{12}N_4O_4$ (336.30): C, 60.71; H, 3.80; N, 16.72%; found: C, 60.94; H, 4.07; N, 16.90%.

***N*-formyl-4-hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridine-3-carbohydrazide (9)**

A solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in formic acid (30 mL) was heated under reflux for 1 h. After cooling, the obtained solid was filtered, dried and crystallized.

Yield 1.65 g (49%), beige solid, crystallized from ethanol, m.p. 296-297°C. IR (KBr, cm^{-1}): 3300 (OH), 2900 (NH), 2750 (CH), 1650, 1600 (C=O), 770 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, CH_3), 7.52-7.56 (m, 3H, phenyl), 8.19-8.22

(m, 3H, phenyl, pyridine), 8.40 (s, 1H, CHO), 10.35 (br, 1H, OH), 10.68 (s, 1H, NH), 11.61 (s, 1H, NH), 12.12 (s, 1H, NH). Analysis: calcd. for $C_{17}H_{14}N_4O_4$ (338.33): C, 60.35; H, 4.13; N, 16.56%; found: C, 60.71; H, 3.73; N, 16.85%.

_N_'-acetyl-4-hydroxy-8-methyl-1-oxo-6-phenyl-2_H_-2,7-naphthyridine-3-carbohydrazide (10)

A solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in acetic acid anhydride (30 mL) was heated under reflux for 2 h. After cooling, the obtained solid was filtered, dried and crystallized.

Yield 1.65 g (47%), beige solid, crystallized from methanol, m.p. 316-318°C. IR (KBr, cm^{-1}): 3300 (OH), 2850 (NH), 1650, 1600 (C=O), 740 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.96 (s, 3H, CH_3), 3.07 (s, 3H, CH_3), 7.52-7.55 (m, 3H, phenyl), 8.19-8.25 (m, 3H, phenyl, pyridine), 10.35 (s, 1H, OH), 10.51 (s, 1H, NH), 10.80 (s, 1H, NH), 11.90 (s, 1H, NH). Analysis: calcd. for $C_{18}H_{16}N_4O_4$ (352.35): C, 61.36; H, 4.58; N, 15.90%; found: C, 61.50; H, 4.25; N, 15.77%.

3-(3,5-Dimethylpyrazole-1-carbonyl)-4-hydroxy-8-methyl-6-phenyl-2_H_-2,7-naphthyridin-1-one (11)

To solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in ethanol (50 mL), pentanedione (0.01 mol) and acetic acid (3 mL) were added. The mixture was refluxed with stirring for 5 h. After cooling, the obtained solid was filtered, dried and crystallized.

Yield 2.69 g (72%), yellow solid, crystallized from ethanol, m.p. 219-220°C. IR (KBr, cm^{-1}): 3500 (OH), 3000 (NH), 1660 (C=O), 1580 (C=N), 770 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 1.88 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 3.03 (s, 3H, CH_3), 6.86 (s, 1H, CH), 7.50-7.52 (m, 3H, phenyl), 8.13-8.17 (m, 3H, phenyl, pyridine), 11.15 (s, 1H, OH), 11.92 (s, 1H, NH). Analysis: calcd. for $C_{21}H_{18}N_4O_3$ (374.39): C, 67.36; H, 4.80; N, 15.02%; found: C, 67.06; H 4.96; N, 15.07%.

4-Phenyl-1-(4-hydroxy-8-methyl-1-oxo-6-phenyl-2_H_-2,7-naphthyridine-3-carbonyl)thiosemicarbazide (12)

To solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in ethanol (50 mL) phenyl isothiocyanate (0.01 mol) was added. The mixture was refluxed with stirring for 6 h. After cooling, the separated solid was filtered, dried and crystallized.

Yield 3.07 g (69%), white solid, crystallized from ethanol, m.p. 327-330°C. IR (KBr, cm^{-1}): 3300 (OH), 2800 (NH), 2300 (NCS), 1650, 1600, 1500, 1350, 1300 (C=O, NH, CN), 820, 700 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.05 (s, 3H, CH_3), 7.15 (s, 1H, phenyl), 7.32-7.35 (m, 2H, phenyl), 7.52-7.60 (m, 5H, phenyl), 8.12-8.21 (m, 4H, phenyl, pyridine, OH), 9.65-9.95 (m, 2H, NH), 11.72 (br, 2H, NH). Analysis: calcd. for $C_{23}H_{19}N_5O_3S$ (445.50): C, 62.01; H, 4.30; N, 15.72%; found: C, 61.82; H, 4.06; N, 15.96%.

4-Phenyl-1-(4-hydroxy-8-methyl-1-oxo-6-phenyl-2_H_-2,7-naphthyridine-3-carbonyl)semicarbazide (13)

To a solution of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** (0.01 mol) in ethanol (30 mL), phenyl isocyanate (0.02 mol) was added. The mixture was refluxed with stirring for 4 h. After cooling, the obtained solid was collected. The obtained solid was filtered, dried and crystallized.

Yield 1.72 g (40%), beige solid, crystallized from ethanol, m.p. 280-282°C. IR (KBr, cm^{-1}): 3350, 3200 (OH), 2900 (NH), 1670, 1580 (C=O), 750, 690 (CH arom.). ^{1}H NMR (DMSO-d_6, δ, ppm): 3.04 (s, 3H, CH_3), 6.93-7.23 (m, 3H, phenyl), 7.46-7.53 (m, 5H, phenyl), 7.95 (s, 1H, phenyl), 8.10-8.15 (m, 2H, phenyl, pyridine), 8.20 (m, 2H, NH, OH), 8.76 (s, 1H, NH), 10.10 (br, 2H, NH). Analysis: calcd. for $C_{23}H_{19}N_5O_4$ (429.44): C, 64.37; H, 4.46; N, 16.31%; found: C, 64.76; H, 4.18; N, 16.37%.

Biology

Anti-proliferative *in vitro* tests were performed at the National Cancer Institute (Bethesda, MD, USA) on 60 different human tumor cell lines, representing nine cancer diseases: leukemia, melanoma, cancers of the breast, lung, brain, colon, prostate, ovary, renal. The cancer cell lines were grown in RPMI 1640 medium containing fetal bovine serum (5%) and L-glutamine (2 mM). After cell inoculation (densities from 5000 to 40000 cells/well), the microtiter plates were incubated (37°C, 5% CO_2, 95% air, 100% humidity) for 24 h. Next, cell lines were fixed *in situ* with trichloroacetic acid to represent a measurement of the cell population for each cell line at the time of the compound addition. Experimental compounds were solubilized in DMSO at 400-fold the desired final maximum test concentration. The samples were stored frozen. The aliquot was thawed and diluted to the appropriate

test concentration with complete medium containing gentamicin (50 µg/mL), prior to use. Following compound addition, the microtiter plates were incubated (37°C, 5% CO_2, 95% air, 100% humidity) for 48 h. Next, cells were fixed *in situ* with cold 50% trichloroacetic acid (50 µL) and incubated at 4°C for 60 min. The supernatant was discarded and the microtiter plates were washed with tap water and dried. The 0.4% solution of sulforhodamine B (100 µL) in 1% acetic acid was added to each well. The plates were incubated at room temperature for 10 min. and next, washed with 1% acetic acid and dried. After solubilization with 10 mM trizma base, the absorbance was read on an automated plate reader at a wavelength of 515 nm. Using 7 absorbance measurements the percentage growth was calculated for each of the compounds. The results were shown as percentage of growth of the treated cells (21-23).

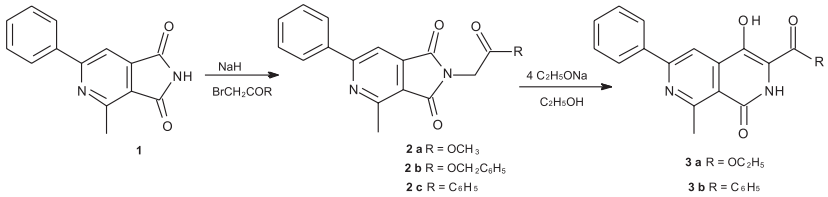
RESULT AND DISCUSSION

Chemistry

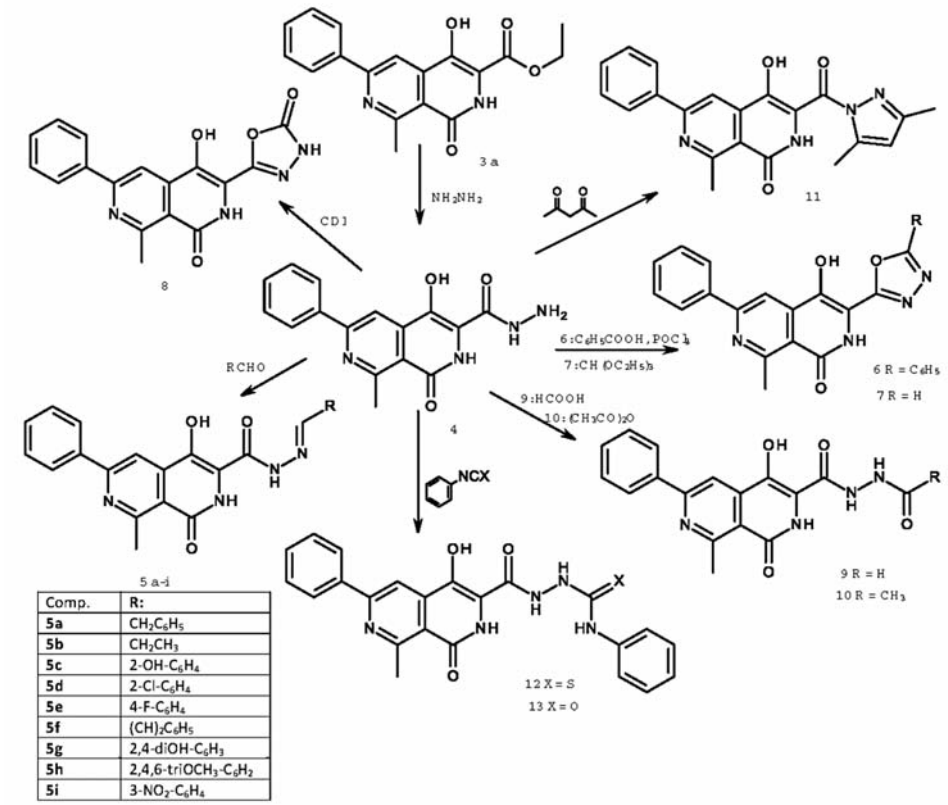
In our previous paper the method of rearrangement of pyrrolo[3,4-*c*]pyridine derivatives to the corresponding 2,7-naphthyridines has been described (17). In the present study, the obtained earlier 4-methyl-6-phenyl-pyrrolo[3,4-*c*]pyridine-1,3-dione **1** was alkylated with methyl or benzyl bromoacetates and bromoacetophenone (Scheme 1), according to the method described by us earlier (17). The new pyrrolo[3,4-*c*]pyridine derivatives **2a**-**c** were isolated with very good yield (72-91%). IR spectra of the obtained compounds **2a**-**c** displayed absorption bands within the range ν = 3000-3400 cm^{-1} characteristic for the NH. ^{1}H NMR spectra contained two-protons singlets at δ = 4.46 ppm for compound **2a**, δ = 5.20 ppm for compound **2b** and δ = 5.26 ppm for compound **2c**, corresponding to protons of the CH_2 group instead of one-proton singlets of pyrrole NH. Stoichiometric amount of sodium ethoxide or sodium methoxide, did not yield the expected 2,7-naphthyridine derivatives. Treatment of pyrrolo[3,4-*c*]pyridine-1,3-dione derivatives with sodium methoxide in a molar ratio of 1 : 4 gave 3-[(carboxymethyl)carbamoyl]-2-alkyl-6-phenylpyridine-4-carboxylic acids. Products isolated in these reactions were described in our previous paper (17). Treatment of pyrrolo[3,4-*c*]pyridine derivatives **2a**-**c** with fourfold excess of sodium ethoxide resulted in the rearrangement to the 2,7-naphthyridine ring. However, the reaction did not yield the expected methyl ester from compound **2a**, and benzyl ester from compound **2b**, but the product of alkoholysis **3a** was isolated. The results of elemental analysis and spectra indicated that the obtained compound **3a** was the same as the ethyl ester synthesized by us earlier from the corresponding ethyl 2-(4-methyl-1,3-dioxo-6-phenyl-pyrrolo[3,4-*c*]pyridin-2-yl)acetate (17). In the ^{1}H NMR spectra of the newly synthesized 3-benzoyl-4-hydroxy-8-methyl-6-phenyl-2H-2,7-naphthyridin-1-one **3b**, two singlets at δ = 8.41 ppm and at δ = 11.10 ppm, corresponding to the OH and NH protons, were observed.

The 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4**, obtained from **3a** according to the method described in our previous paper (18), was found to be useful as the key intermediate for further synthesis. The reactions were illustrated in Scheme 2. The synthesis of Schiff bases **5a**-**i** involved the reaction between appropriate aldehydes and hydrazide **4** in a presence of catalytic amount of indium (III) trifluoromethanesulfonate. In the ^{1}H NMR spectra of obtained Schiff bases the two-protons signal at δ = 4.51 ppm due to the NH_2 group, which was observed in the ^{1}H NMR spectra of hydrazide **4**, disappeared. The appearance of the signals between δ = 6.35 and 7.51 ppm indicates the formation of imines (CH=N).

1,3,4-Oxadiazole derivatives **6**–**8** were produced as the products of cyclocondensation. 4-Hydroxy-8-methyl-6-phenyl-3-(5-phenyl-1,3,4-oxadiazol-2-yl)-2*H*-2,7-naphthyridin-1-one **6** was synthesized from hydrazide **4** with benzoic acid, in the presence of an excess of phosphorous oxychloride. 4-Hydroxy-8-methyl-3-(1,3,4-oxadiazol-2-yl)-6-phenyl-2*H*-2,7-naphthyridin-1-one **7** was obtained from the hydrazide **4** and an equimolar amount of



Scheme 1. Synthesis of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid derivatives



Comp.	R:
5a	$CH_2C_6H_5$
5b	CH_2CH_3
5c	2-OH-C_6H_4
5d	2-Cl-C_6H_4
5e	4-F-C_6H_4
5f	$(CH)_2C_6H_5$
5g	2,4-diOH-C_6H_3
5h	2,4,6-triOCH$_3$-C_6H_2
5i	3-NO_2-C_6H_4

Scheme 2. Synthesis of 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine derivatives

triethyl orthoformate. In the reaction of hydrazide **4** with 1,1-carbonyldiimidazole, 5-(4-hydroxy-8-methyl-1-oxo-6-phenyl-2H-2,7-naphthyridin-3-yl)-3*H*-1,3,4-oxadiazol-2-one **8** was isolated.

In the next synthesis, carbohydrazide derivatives were produced. The reaction of hydrazide **4** with formic acid or acetic acid anhydride resulted in the formation of *N*-formyl-4-hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridine-3-carbohydrazide **9** and *N*'-acetyl-4-hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridine-3-carbohydrazide **10**, respectively. The number of signals for the protons in the ^{1}H NMR spectra of obtained compounds is in good agreement with their structures.

Cyclocondensation of hydrazide **4** with pentanedione in the presence of catalytic amount of acetic acid resulted in the formation of 3-(3,5-dimethylpyrazole-1-carbonyl)-4-hydroxy-8-methyl-6-phenyl-2*H*-2,7-naphthyridin-1-one **11** in good yield (72%). ^{1}H NMR spectra exhibited three three-protons singlets at δ = 1.88 ppm, δ = 2.05 ppm, and δ = 3.03 ppm for the methyl groups.

The reaction of hydrazide **4** with phenyl isocyanate or phenyl isothiocyanate in boiling ethanol gave 4-phenyl-1-(4-hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridine-3-carbonyl)semicarbazide **13** and 4-phenyl-1-(4-hydroxy-8-methyl-1-oxo-6-phenyl-2*H*-2,7-naphthyridine-3-carbonyl) thiosemicarbazide **12**, respectively. ^{1}H NMR spectra of the obtained compounds contain five additional signals for the aromatic protons at δ = 7.46-7.60 ppm and one more signal corresponding to proton of NH. Additionally, IR spectrum of the thiosemicarbazide **12** contains among other absorption bands, those within the range of ν = 2300 cm^{-1} characteristic for the NC=S group.

Biology

In our previous works, 8-ethoxy-4-hydroxy-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid derivatives were evaluated for their antitumor activity *in vitro* (17, 18). The tested compounds demonstrated variable antitumor activity. Among all derivatives, the hydrazide derivatives showed the better antiproliferative activity *in vitro*. The 8-ethoxy-4-hydroxy-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2,4-dihydroxy-bezylidene)-hydrazide (24) and 8-ethoxy-4-

Table 1. *In vitro* percent growth of some selected tumor cell lines caused by the tested compounds.

Compd.	Panel	Tumor cell line	Percent growth
5a	Leukemia	SR	46.06
	Leukemia	HL-60(TB)	76.79
	Leukemia	K-562	76.63
5b	Leukemia	MOLT-4	52.92
	Leukemia	CCRF-CEM	68.37
	Leukemia	K-562	44.47
	Leukemia	SR	52.96
	Colon Cancer	HCT-15	67.73
	Renal Cancer	A498	65.89
	Renal Cancer	CAKI-1	39.95
	Renal Cancer	UO-31	66.57
5g	Leukemia	CCRF-CEM	49.97
	Non-small Cell Lung Cancer	NCI-H322M	67.90
	Renal Cancer	A498	68.29

Data obtained from the NCI's *in vitro* human tumor cell screen.

hydroxy-3-(1,3,4-oxadiazol-2-yl)-6-phenyl-2*H*-2,7-naphthyridin-1-one (25) were active against most of the 60 different subpanel tumor cell lines. The results have been encouraging to the preparation of new hydrazide derivatives.

Eight of the newly synthesized compounds: **2a, 2c, 5a, 5b, 5f, 5g, 6, 11** were qualified by the National Cancer Institute in Bethesda (USA) for antiproliferative *in vitro* screening. These compounds were tested against 60 different human tumor cell lines, representing leukemia, melanoma, and breast, lung, colon, ovary, renal, prostate, central nervous system cancers in a single dose of 10 μmol. Antitumor activity was reported as percentage of growth of the treated cells. A value of 100 means no growth inhibition and a value of 0 means no net growth over the course of the experiment. Values below 0 designate percentage of lethality.

Unfortunately, compounds **2a**, **2c**, **5f**, **6** and **11** were inactive (growth higher than 50% in all cell lines). Only Schiff bases **5a**, **5b**, **5g** showed the moderate growth inhibitory activity against a few of the cell lines. The most interesting results are depicted in Table 1. The most sensitive to their antitumor activity were found to be human leukemia and renal cancer cells.

CONCLUSIONS

The aim of the present research was to synthesize the novel pyrrolo[3,4-*c*]pyridines and their rearrangement to the corresponding 2,7-naphthyridines (Scheme 1). Next step of this work was to obtain 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid hydrazide **4** derivatives of various chemical structure, according to the syntheses illustrated in Scheme 2. Twenty-one new compounds were isolated as the result of these reactions. Their structures were confirmed by IR, NMR, MS spectra and elemental analysis. Eight of the prepared compounds were evaluated against the 60 different human tumor cell lines for their antiproliferative activity *in vitro*. Among the tested compounds the Schiff bases: 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid phenethylidene-hydrazide **5a**, 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid propylidene-hydrazide **5b**, and 4-hydroxy-8-methyl-1-oxo-6-phenyl-1,2-dihydro-2,7-naphthyridine-3-carboxylic acid (2,4-dihydroxy-benzylidene)-hydrazide **5g** showed the moderate antitumor activity *in vitro*.

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ANTICANCER ACTIVITY OF NEWLY SYNTHESIZED TRIAZOLOPYRIMIDINE DERIVATIVES AND THEIR NUCLEOSIDE ANALOGS

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Abstract: New substituted triazolopyrimidne derivatives were synthesized starting from 1,2,3-triazolo-4-carboxamide derivative. The *N*- and *S*-glycoside derivatives of the synthesized triazolopyrimidine ring system as well as their acyclic sugar analogs were also synthesized. The cytotoxicity and *in vitro* anticancer evaluation of the prepared compounds have been assessed against three different human tumor cell lines including human breast MCF-7, lung A549 and colon HCT116 cancer cell lines. The results revealed that the prepared compounds exert their actions in MCF-7 and A549. MCF-7 cells are more sensitive to the tested compounds than the other cell lines. Compounds **2**, **3**, **9** and **10** revealed promising anticancer activities compared to the activity of the commonly used anticancer drug, doxorubicin in both MCF-7 and A549 cell lines.

Keywords: triazolopyrimidine, glycosides, anticancer, MCF-7, A549, HCT116

Although there have been great advances in the detection and treatment of cancer, it remains one of the greatest medical challenges, with the incidence of some malignancies continuing to increase (1). For many tumor types, established treatments such as cytotoxic chemotherapy and radiotherapy provide only transient therapeutic benefits despite severe side effects (2). Therefore, the need for better treatments has stimulated research to develop new efficient chemotherapeutic agents for management of cancer.

The search for new heterocyclic compounds and novel methods for their synthesis is a major topic in contemporary medicinal chemistry (3-6) representing one of the ways to face cancer diseases. Pyrimidines has gained considerable attention because of their role in biological systems, particularly in nucleic acids, which contain pyrimidines and purines as the main nucleobases. It has been noticed that introduction of an additional ring to the pyrimidines core tends to exert profound influence in conferring novel biological activities in these molecules (7-9). Consequently, the aza analogs of purines, mainly the triazolo[x,y-z]pyrimidines, also are important (8). The study of compounds incorporating the triazolopyrimidine has been developed due to their varied effects in diverse domains. Triazolopyrimidines (TPs), a subtype of purine analogs, have been the subject of chemical and biological studies due to their interesting pharmacology including antihypertensive, cardiac stimulant, antimalarial, antifungal, anti-HBV, antimicrobial, anticancer, antipyretic, analgesic, antiinflammatory, potential herbicidal, and leishmanicidal activities (10–25). In addition, triazolopyrimidines are versatile ligands and their derived coordination compounds can be considered as model systems for metal–ligand interactions observed in biological systems (17, 26). The simple molecule of Trapidil, the most widely known triazolopyrimidine derivative acts as a platelet-derived growth factor antagonist and as a phosphodiesterase inhibitor (11).

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Cevipabulin and its analogs represent a class of triazolo[1,5-a]pyrimidines and were proved to be potent anticancer agents with an unique mechanism of action in promoting tubulin polymerization (17).

On the other hand, the acyclic nucleoside analogs with modification of both the glycon part and the heterocyclic core have stimulated extensive research as biological inhibitors (27-29). Nucleosides and their analogs have been known to possess antibiotic, antiviral, and antitumor activity (22–33). We have been interested in our previous work in developing new bioactive modified heterocyclic sugar derivatives by attachment of sugar moieties to newly synthesized heterocycles (34-39) in an ongoing search for new compounds with potential biological activity.

In the same direction and in continuing effort to find more potent and selective anticancer compounds, herein, we synthesized triazolopyrimidine compounds, their glycosides and acyclic sugar derivatives. Their biological activities against three different human breast MCF-7, lung A549 and colon HCT116 cancer cell lines were evaluated.

EXPERIMENTAL

Chemistry

All melting points were measured on Electro thermal IA 9000 series digital melting point apparatus. The IR spectra were recorded in potassium bromide discs on a PyeUnicam SP 3300 and Shimadzu FT IR 8101 PC infrared spectrophotometers. The NMR spectra were recorded at 270 MHz on a Varian Mercury VX-300 NMR spectrometer. ^{1}H NMR (300 MHz) and ^{13}C NMR (75.5 MHz) were run in deuterated chloroform ($CDCl_3$) or dimethyl sulfoxide (DMSO-d_6). Chemical shifts were related to that of the solvent. Mass spectra were recorded on a Shimadzu GCMS-QP1000 EX mass spectrometer at 70 eV. Elemental analyses were carried out at the Micro analytical Centre of Cairo University, Giza, Egypt. All reactions were followed by TLC using silica gel, aluminum sheets 60 F_{254}, (Merck). The anticancer screening was run in the United States National Institute of Health (NIH)/National Cancer Institute (NCI). Compounds **1** and **2** were prepared according to a previously reported method (40-43).

3-(4-Methoxybenzyl)-5-thioxo-5,6-dihydro-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(4H)-one (3)

To a solution of the carboxamide derivative **2** (10 mmol) in dimethylformamide (30 mL), sodium hydroxide (12 mL, 10%) was added dropwise at 0°C, the reaction mixture was stirred at 0°C for one hour, then carbon disulfide (14 mmol) was added and the temperature of the reaction was raised up to room temperature, then refluxed in water bath for 8 h. The solvent was distilled off under vacuum and the residue was dissolved in ice-water and then acidified with diluted hydrochloric acid to afford compound **3**.

3-(4-Methoxybenzyl)-3H-[1,2,3]triazolo[4,5-d] pyrimidin-7(6H)-one (4)

A mixture of the triazole derivative **2** (10 mmol) and triethyl orthoformate (20 mmol) in ethanol (25 mL) was heated at 100°C for 8 h. The excess of triethyl orthoformate was removed under vacuum and the residue was dissolved in ethanol, then allowed to stand at room temperature overnight to afford compound **4**.

5-(4-Chlorophenyl)-3-(4-methoxybenzyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (5)

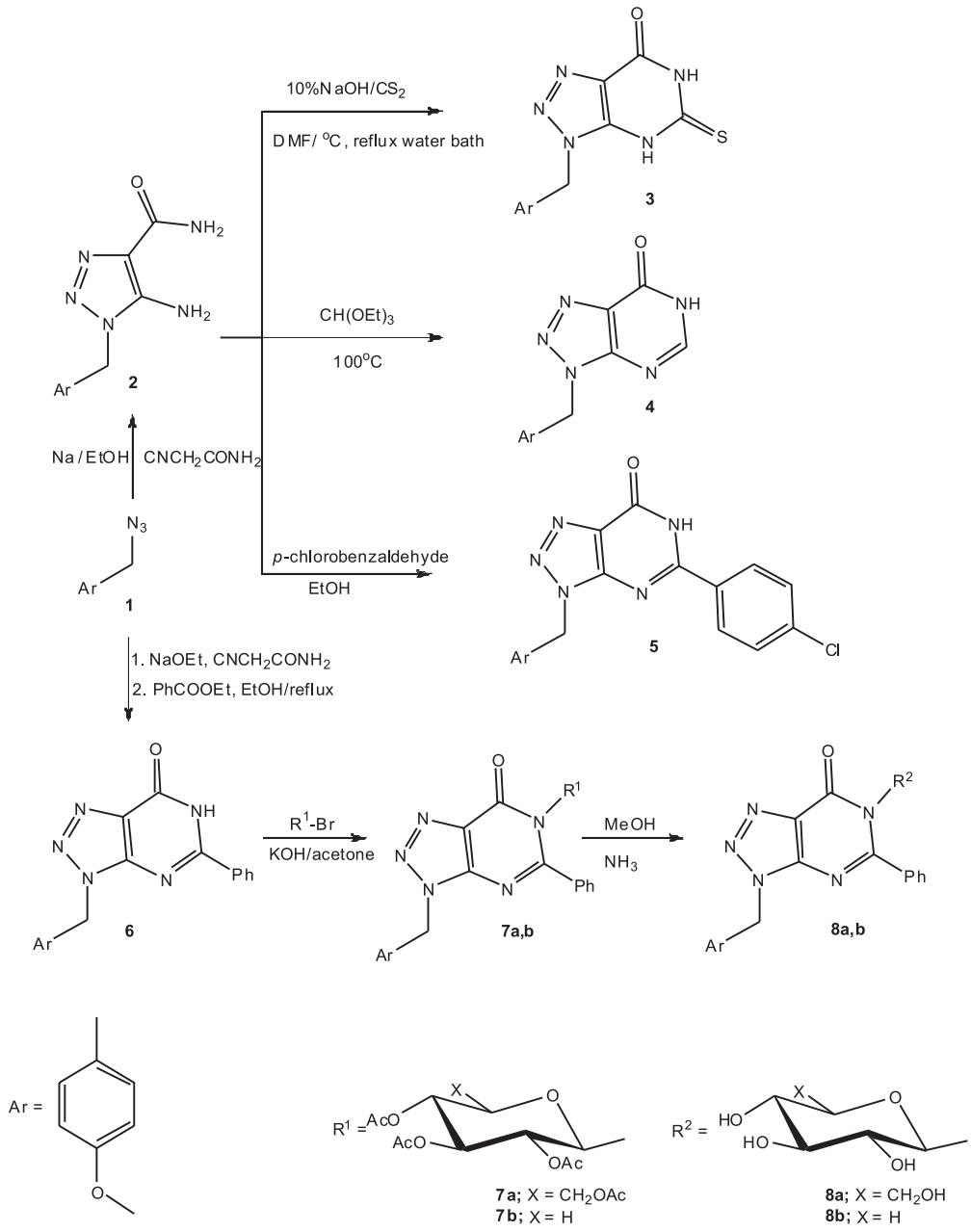
A solution of the carboxamide derivative **2** (10 mmol) and *p*-chlorobenzaldehyde (10 mmol) in ethanol (30 mL) was heated under reflux for 8 h. The solvent was distilled off *in vacuo* and the residue was recrystallized from ethanol-water mixture (1 : 1) to afford compound **5**.

3-(4-Methoxybenzyl)-5-phenyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (6)

To a stirred solution of EtONa (2.76 g, 0.12 g atom of Na) in 30 mL of absolute EtOH, cyanacetamide (2.51 g, 3 mmol) was added. The mixture was refluxed for 0.5 h, then a solution of the azide **1** (3 mmol) and ethyl benzoate (3 mmol) in absolute EtOH (10 mL) was added drop by drop and the mixture was refluxed for 6 h, then cooled and concentrated under reduced pressure. To the residue, water (20 mL) was added and the solution was acidified with 4 M acetic acid to pH = 5. The precipitated solid was filtered and crystallized from ethanol.

6-(Glucosyl)-3-(4-methoxybenzyl)-5-phenyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (7a,b)

To a solution of compound **6** (5 mmol) in aqueous potassium hydroxide [(10 mmol in distilled water (16 mL)] was added a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galacto, glucopyranosyl bromide, or 2,3,4-tri-*O*-acetyl-α-D-xylopyranosyl bromide (5 mmol) in acetone (20 mL). The reaction mixture was stirred at room temperature for 10-12 h (TLC). The solvent was evaporated under reduced pressure at 40°C and the residue was washed with distilled water to remove potassium bromide formed. The product was dried, and crystallized from ethanol to give compounds **7a**,**b**, respectively.



Scheme 1. Synthesis of triazolopyrimidine derivatives.

3-(4-Methoxybenzyl)-6-(D-glucopyranosyl)-5-phenyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (8a,b)

Dry gaseous ammonia was passed through a solution of a protected nucleoside **7a**,**b** (0.3 mmol) in dry methanol (12 mL) at 0°C for 1 h, and then the mixture was stirred at 0°C for ca. 5 h. The solvent was evaporated under reduced pressure at 40°C to give a solid residue, which was crystallized from ethanol to give compounds **8a** and **8b**, respectively.

5-(2,3,4,6-Tetra-*O*-acetyl-D-glucopyranosylthio)-3-(4-methoxybenzyl)-3*H*-[1,2,3]triazolo[4,5-d]pyrimidin-7(6*H*)-one (9)

To a solution of compound **3** (5 mmol) in aqueous potassium hydroxide [(10 mmol in distilled water (16 mL)] was added a solution of 2,3,4,6-tetra-*O*-acetyl-α-glucopyranosyl bromide (5 mmol) in acetone (20 mL). The reaction mixture was stirred at room temperature for 8 h (TLC). The

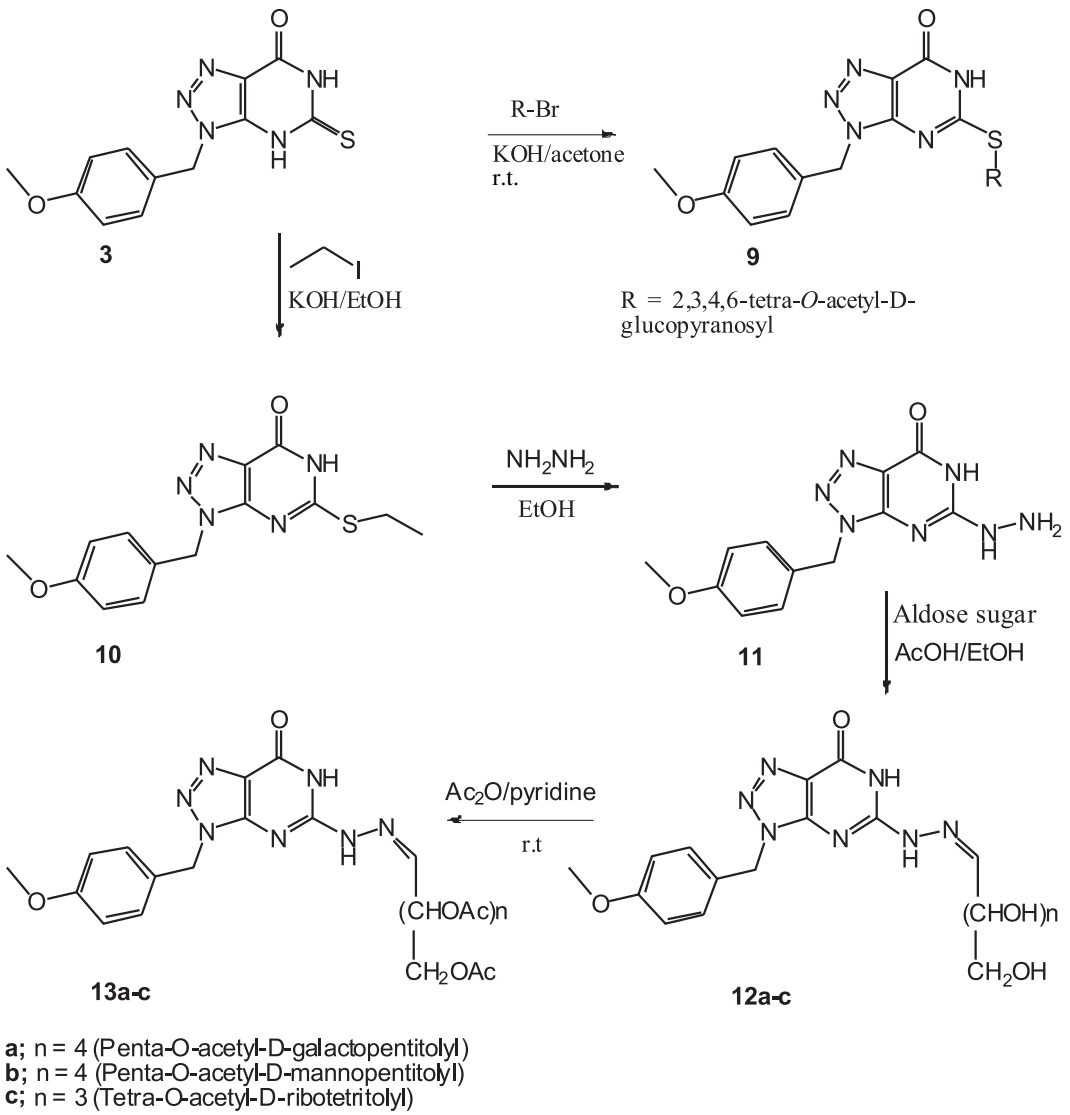
solvent was evaporated under reduced pressure at 40°C and the residue was washed with distilled water to remove potassium bromide formed. The product was dried, and crystallized from ethanol to give compound **9**.

5-(Ethylthio)-3-(4-methoxybenzyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (10)

To a solution of the pyrimidine thione **3** (10 mmol) in 50 mL ethanol, potassium hydroxide (10 mmol) in water (5 mL) was added and a greenish precipitate was formed. Ethyl iodide (10 mmol) was added and a white precipitate was formed. The reaction mixture was stirred at room temperature for 4 h and refluxed for another 5 h. The resulting precipitate was filtered off and crystallized from ethanol.

5-Hydrazinyl-3-(4-methoxybenzyl)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (11)

A solution of compound **10** (10 mmol) and hydrazine hydrate (15 mmol) in ethanol was heated under reflux for 6 h. The solution was cooled and the resulting precipitate was filtered and crystallized from ethanol.



3 9 10 11 13a-c 12a-c

a; n = 4 (Penta-O-acetyl-D-galactopentitolyl)
b; n = 4 (Penta-O-acetyl-D-mannopentitolyl)
c; n = 3 (Tetra-O-acetyl-D-ribotetritolyl)

Scheme 2. Synthesis of triazolopyrimidine sugar derivatives

Table 1. *In vitro* cytotoxicity activity of the synthesized compounds in three human cancer cell lines as measured with SRB assay.

Compound	IC_{50} (μg/mL)		
	MCF-7	A549	HCT116
Doxorubicin	2.90 ± 0.27	4.30 ± 0.40	4.80 ± 0.50
DMSO	N.A.	N.A.	N.A.
2	4.80 ± 0.55	8.20 ± 0.78	75.40 ± 8.80
3	2.80 ± 0.33	4.20 ± 0.50	75.00 ± 7.30
4	N.A.	N.A.	N.A.
6	14.70 ± 1.75	33.20 ± 5.00	N.A.
7a	N.A.	N.A.	N.A.
9	3.90 ± 0.42	8.00 ± 0.83	80.70 ± 9.30
10	3.60 ± 0.39	6.50 ± 0.67	66.80 ± 7.50
11	22.70 ± 2.50	28.00 ± 3.29	88.50 ± 9.40
12a	N.A.	N.A.	N.A.

Data were expressed as the mean ± standard error (SE) of six independent experiments. N.A. is no activity.

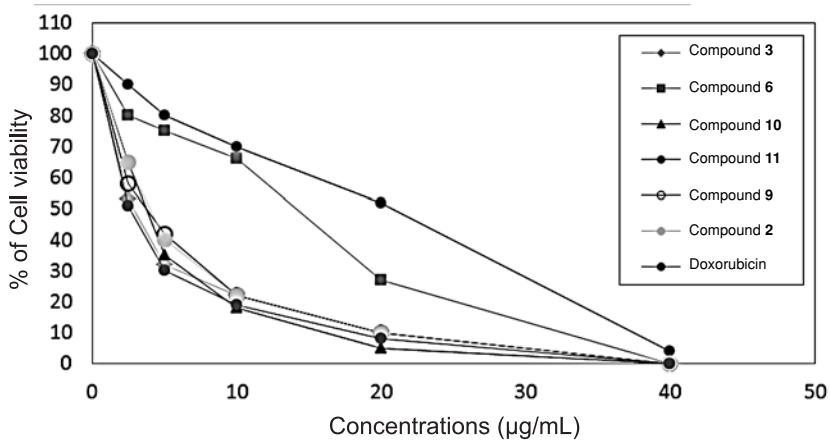


Figure 1. Effect of treatment with various concentrations of prepared compounds on breast MCF-7 cell line cytotoxicity (IC_{50}) as measured with SRB method

(Z)-3-(4-Methoxybenzyl)-5-(hydrazinylsugar)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (12a-c)

General procedure

To a well-stirred solution of the respective monosaccharide (0.01 mol) in water (2 mL), and glacial acetic acid (0.2 mL) was added the hydrazine derivative **11** (10 mmol) in ethanol (15 mL). The mixture was heated under reflux for 4-6 h (TLC) and the resulting solution was concentrated and left to cool. The precipitate formed was filtered off, washed with water, then dried and crystallized from ethanol-DMF (2 : 1, v/v).

(Z)-3-(4-Methoxybenzyl)-5-(per-*O*-acetylhydrazinylsugar)-3H-[1,2,3]triazolo[4,5-d]pyrimidin-7(6H)-one (13a-c)

General procedure

To a solution of the hydrazinyl sugar derivative **12a-c** (5 mmol) in pyridine (10 mL) was added acetic anhydride (6 mmol) and the mixture was stirred at room temperature for 7 h. The resulting solution was poured onto crushed ice, and the product that separated out was filtered off, washed with sodium hydrogen carbonate and water, then dried to afford compounds **13a-c**.

Table 2. Physical and analytical data of the synthesized compounds.

Compd. no.	M.p. (°C)	Yield (%)	Mol. formula	Analysis (%) calc. / found		
				C	H	N
3	36-238	77	$C_{12}H_{11}N_5O_2S$	49.82 49.59	3.83 3.69	24.21 24.14
4	155-156	76	$C_{12}H_{11}N_5O_2$	56.03 55.89	4.31 4.26	27.22 27.17
5	190-192	79	$C_{18}H_{14}ClN_5O_2$	58.78 58.68	3.84 3.80	19.04 19.18
6	261-263	78	$C_{18}H_{15}N_5O_2$	64.86 64.70	4.54 4.42	21.01 20.90
7a	148-150	58	$C_{32}H_{33}N_5O_{11}$	57.92 57.59	5.01 4.88	10.55 10.39
7b	151-153	72	$C_{29}H_{29}N_5O_9$	58.88 58.70	4.94 4.85	11.84 11.75
8a	199-200	66	$C_{24}H_{25}N_5O_7$	58.18 58.05	5.09 4.90	14.13 13.98
8b	200-202	62	$C_{23}H_{23}N_5O_6$	59.35 59.18	4.98 5.05	15.05 14.82
9	> 300	73	$C_{26}H_{29}N_5O_{11}S$	50.40 50.62	4.63 4.63	11.30 11.19
10	223-225	74	$C_{14}H_{15}N_5O_2S$	52.98 52.81	4.76 4.72	22.07 21.98
11	296-298	79	$C_{12}H_{13}N_7O_2$	50.17 50.05	4.56 4.45	34.13 34.02
12a	153-156	79	$C_{18}H_{23}N_7O_7$	48.11 47.92	5.16 5.10	21.82 21.69
12b	158-160	77	$C_{18}H_{23}N_7O_7$	48.11 48.02	5.16 5.12	21.82 21.71
12c	156-159	75	$C_{17}H_{21}N_7O_6$	48.69 48.55	5.05 5.02	23.38 23.25
13a	188-191	79	$C_{28}H_{33}N_7O_{12}$	50.99 50.80	5.04 4.91	14.86 14.71
13b	193-195	80	$C_{28}H_{33}N_7O_{12}$	50.99 50.75	5.04 4.92	14.86 14.69
13c	193-195	80	$C_{25}H_{29}N_7O_{10}$	51.11 50.95	4.98 4.92	16.69 16.51

Biological evaluation

Chemicals

Fetal bovine serum (FBS) and L-glutamine were obtained from Gibco Invitrogen Company (Scotland, UK). Dulbecco's modified Eagle's (DMEM) medium was provided from Cambrex (New Jersey, USA). Dimethyl sulfoxide (DMSO), doxorubicin, penicillin, and streptomycin were obtained from Sigma Chemical Company (Saint Louis, MO, USA).

Cell lines and culturing

Anticancer activity screening for the tested compounds utilizing three different human tumor cell lines including human breast MCF-7, lung A549 and colon HCT116 cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal calf serum (GIBCO), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C in humidified atmosphere containing 5% CO_2. Cells at a concentration of 0.50×10^6 were grown in a 25 cm^2 flask in 5 mL of complete culture medium.

In vitro cytotoxicity assay

The cytotoxicity activity was measured *in vitro* using the sulforhodamine-B stain (SRB) assay

according to the previously reported standard procedure (44). Cells were inoculated in 96-well microtiter plate (10^4 cells/ well) for 24 h before treatment with the tested compounds to allow attachment of cell to the wall of the plate. Test compounds were dissolved in DMSO at 1 mg/mL immediately before use and diluted to the appropriate volume just before addition to the cell culture. Different concentrations of tested compounds and doxorubicin were added to the cells. Six wells were prepared for each individual dose. Cells were incubated with the compounds for 48 h, at 37°C and in atmosphere of 5% CO_2. After 48 h cells were fixed, washed, and stained for 30 min with 0.4% (w/v) SRB dissolved in 1% acetic acid. Unbound dye was removed by four washes with 1% acetic acid, and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between surviving fraction and drug concentration is plotted to get the survival curve for each cell line after the specified time. The concentration required for 50% inhibition of cell viability (IC_{50}) was calculated and the results are given in Table 1. The results were compared to the antiproliferative effects of the reference control - doxorubicin (45).

Statistical analysis

The results are reported as the mean ± standard error (SE) for at least six times experiments.

RESULTS AND DISCUSSION

Chemistry

In this investigation, the starting carboxamide key derivative **2** was synthesized according to a pre-

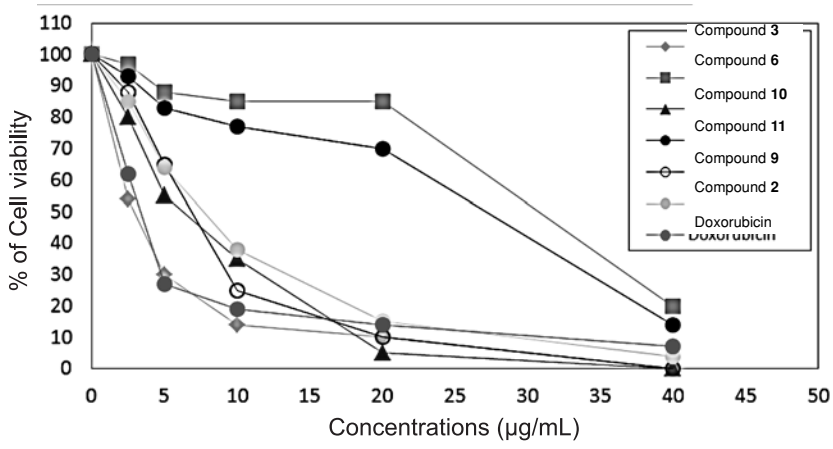


Figure 2. Effect of treatment with various concentrations of prepared compounds on lung A549 cell line cytotoxicity (IC_{50}) as measured with SRB method

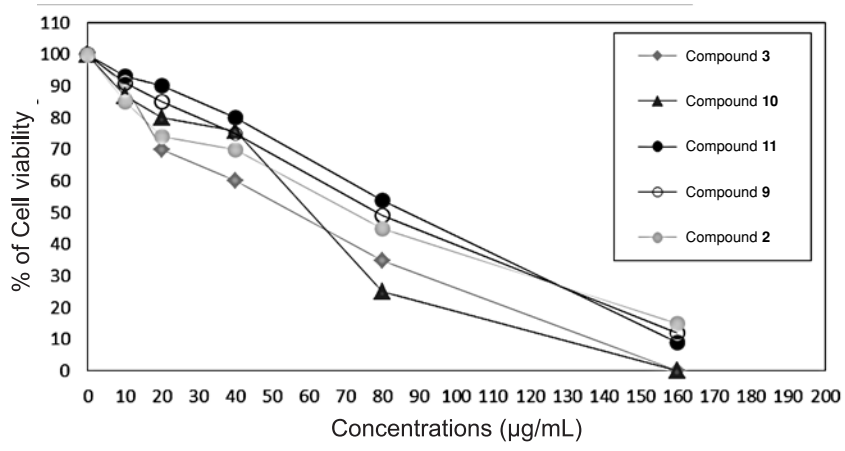


Figure 3. Effect of treatment with various concentrations of prepared compounds on colon HCT116 cell line cytotoxicity (IC_{50}) as measured with SRB method

Table 3. Spectral data for the synthesized compounds.

Comp. no.	IR [KBr; ν cm^{-1}], ^{1}H NMR [δ, ppm], ^{13}C NMR [δ, ppm], MS [m/z]
3	IR: 3285 (NH), 1662 (C=O). ^{1}H NMR (DMSO-d_6): 3.71 (s, 3H, OCH_3), 5.21 (s, 2H, CH_2), 6.90 (m, 2H, Ar-H), 7.26 (d, 2H, *J* = 7.6 Hz, Ar-H), 12.57 (s, 1H, NH), 13.95 (bs, 1H, NH). ^{13}C NMR (DMSO-d_6): 50.0 (CH_2), 56.5 (CH_3), 115.9-154.7 (Ar-C and pyrimidyl C), 159.98 (C=O), 175.9 (C=S). MS: *m*/*z*: 289 [M^+].
4	IR: 3250 (NH), 1658 (C=O). ^{1}H NMR (DMSO-d_6): 3.81 (s, 3H, OCH_3), 5.30 (s, 2H, CH_2), 6.95 (d, 2H, Ar-H), 7.22 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.92 (s, 1H, pyrimidine H-2), 9.28 (s, 1H, NH). MS: *m*/*z*: 257 [M^+].
5	IR: 3290 (NH), 1661 (C=O). ^{1}H NMR (DMSO-d_6): 3.80 (s, 3H, OCH_3), 5.31 (s, 2H, CH_2), 6.95 (m, 2H, Ar-H), 7.27 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.48 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.66 (d, 2H, *J* = 7.6 Hz, Ar-H), 9.28 (s, 1H, NH). MS: *m*/*z*: 368 [M^+].
6	IR: 3277 (NH), 1664 (C=O). ^{1}H NMR (DMSO-d_6): 3.72 (s, 3H, OCH_3), 5.32 (s, 2H, CH_2), 6.93 (m, 2H, Ar-H), 7.39 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.59-7.64 (m, 3H, Ar-H), 7.98-8.19 (m, 2H, Ar-H), 12.83 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): 49.9 (CH_2), 57.5 (CH_3), 115.8-151.9 (Ar-C and pyrimidyl C), 158.8 (C=O), 159.9 (pyrimidyl C-2). MS: *m*/*z*: 333 [M^+].
7a	IR: 1735 (C=O), 1668 (C=O). ^{1}H NMR ($CDCl_3$): 1.77, 1.95, 2.02, 2.06 (4s, 12H, 4 CH_3CO), 3.71 (s, 3H, OCH_3), 4.03 (m, 1H, H-5), 4.09 (dd, 1H, $J_{6,6'}$ = 11.4 Hz, $J_{5,6}$ = 2.8 Hz, H-6), 4.16 (m, 1H, H-6'), 4.56 (t, 1H, $J_{3,4}$ = 9.3 Hz, H-4), 5.12 (dd, 1H, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 5.36 (t, 1H, $J_{2,3}$ = 9.6 Hz, H-2), 5.41 (s, 2H, CH_2), 5.87 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 6.92 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.38 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.63 (m, 3H, Ar-H), 8.19 (m, 2H, Ar-H). ^{13}C NMR ($CDCl_3$): 20.67, 20.75, 20.79, 20.89 (4CH_3CO), 50.2 (CH_2), 56.3 (OCH_3), 62.2 (C-6), 67.7 (C-4), 68.6 (C-3), 70.7 (C-2), 72.5 (C-5), 93.8 (C-1), 114.6-152.7 (Ar-C and pyrimidyl 2C), 159.7 (C=N), 162.8 (C=O), 169.6, 169.9, 170.1, 140.4 (4CH_3C=O).
7b	IR: 1738 (C=O), 1660 (C=O). ^{1}H NMR ($CDCl_3$): 1.80, 1.92, 2.04 (3s, 9H, 3 CH_3CO), 3.71 (s, 3H, OCH_3), 4.09 (dd, 1H, *J* = 11.4 Hz, *J* = 2.8 Hz, H-5), 4.18 (m, 1H, H-5'), 4.78 (t, 1H, $J_{3,4}$ = 9.3 Hz, H-4), 4.95 (dd, 1H, $J_{2,3}$ = 9.6 Hz, $J_{3,4}$ = 9.3 Hz, H-3), 5.11 (t, 1H, $J_{2,3}$ = 9.6 Hz, H-2), 5.35 (s, 2H, CH_2), 5.88 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 6.93 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.39 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.63 (m, 3H, Ar-H), 8.18 (m, 2H, Ar-H); ^{13}C NMR ($CDCl_3$): 20.6, 20.7, 20.8 (3CH_3CO), 50.2 CH_2), 56.3 (OCH_3),62.2 (C-5), 67.8 (C-4), 69.5 (C-3), 70.9 (C-2), 94.1 (C-1), 114.5-152.8 (Ar-C and pyrimidyl 2C), 159.5 (C=N), 162.8 (C=O), 169.5, 169.9, 140.5 (3CH_3C=O).
8a	IR: 3475-3440 (OH), 1662 (C=O). ^{1}H NMR (DMSO-d_6): 3.72 (s, 3H, OCH_3), 3.88-4.05 (m, 2H, H-6,6'), 4.27 (m, 1H, H-5), 4.77-5.10 (m, 3H, H-4,3 and OH), 5.31 (m, 3H, CH_2 and OH), 5.38 (m, 1H, H-2), 5.60 (m, 1H, OH), 5.64 (s, 1H, OH), 5.86 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 6.93 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.37 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.68 (m, 3H, Ar-H), 8.17 (m, 2H, Ar-H).
8b	IR: 3480-3445 (OH), 1662 (C=O). ^{1}H NMR (DMSO-d_6): 3.74 (s, 3H, OCH_3), 3.85-4.04 (m, 2H, H-5,5'), 4.77-5.03 (m, 2H, H-4,3), 5.15-5.28 (m, 3H, CH_2 and OH), 5.35 (m, 1H, H-2), 5.62 (m, 1H, OH), 5.66 (s, 1H, OH), 5.88 (d, 1H, $J_{1,2}$ = 10.2 Hz, H-1), 6.94 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.38 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.69 (m, 3H, Ar-H), 8.17 (m, 2H, Ar-H).
9	IR: 1695 (C=O), 1734 (C=O). ^{1}H NMR ($CDCl_3$): 1.89, 1.93, 1.98, 2.03 (4s, 12 H, CH_3CO), 3.77 (s, 3H, OCH_3), 3.99 (m, 1H, H-5), 4.05 (dd, 1H, *J* = 2.8, *J* = 11.0, H-6), 4.15 (dd, *J* = 2.8 Hz, *J* = 11.4 Hz, 1H, H-6'), 4.94 (t, $J_{3,4}$ = 9.3 Hz, 1H, H-4), 5.20-5.27 (m, 3H, CH_2 and H-3), 5.33 (t, 1H, $J_{2,3}$ = 9.6 Hz, H-2), 5.77 (d, $J_{1,2}$ = 10.2 Hz, 1H, H-1), 6.94 (d, 2H, *J* = 8.5 Hz, Ar-H), 7.40 (d, 2H, *J* = 8.4 Hz, Ar-H), 12.02 (bs, 1H, NH); ^{13}C NMR ($CDCl_3$): 20.05, 20.18, 20.66, 20.82 (4CH_3CO), 50.5 CH_2), 56.8 (OCH_3), 62.1 (C-6), 7.5 (C-4), 68.9 (C-3), 70.7 (C-2), 72.5 (C-5), 90.2 (C-1), 116.6-154.4 (Ar-C and pyrimidyl 2C), 158.9 (C=N), 162.6 (C=O), 169.1, 169.8, 170.2, 170.5 (4CH_3C=O).

Table 3. cont.

Comp. no.	IR [KBr; ν cm^{-1}], ^{1}H NMR [δ, ppm], ^{13}C NMR [δ, ppm], MS [m/z]
10	IR: 3275 (NH), 1664 (C=O). ^{1}H NMR (DMSO-d_6): 1.33 (t, 3H, *J* = 5.2 Hz, CH_3CH$_2$), 3.72 (s, 3H, OCH$_3$), 4.42 (q, 2H, *J* = 5.2 Hz, CH$_3$CH_2), 5.61 (s, 2H, CH$_2$),6.92 (d, 2H,*J* = 7.6 Hz,Ar-H), 7.34 (d, 2H, *J* = 7.6 Hz, Ar-H), 12.87 (s, 1H, NH); ^{13}C NMR (DMSO-d_6): 15.6 (CH$_3$), 25.4 (CH$_2$), 49.9 (CH$_2$), 55.6 (OCH$_3$), 114.6-156.3 (Ar-C and pyrimidyl 2C), 159.6 (C=N), 162.7 (C=O). MS: *m*/*z*: 317 [M$^+$].
11	IR: 3340 and 3305 (NH$_2$), 3255 (NH), 1668 (C=O). ^{1}H NMR (DMSO-d_6): 3.73 (s, 3H, OCH$_3$), 5.32 (s, 2H, CH$_2$),5.68 (bs, 2H, NH$_2$), 6.91 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.43 (d, 2H, *J* = 7.6 Hz, Ar-H), 10.02 (bs, 1H, NH), 12.63 (bs, 1H, NH). MS: *m*/*z*: 287 [M$^+$].
12a	IR: 3495 (OH), 3269 (NH), 1664 (C=O). ^{1}H NMR (DMSO-d_6): 3.31-3.43 (m, 2H, H-6, H-6'), 3.61-3.65 (m, 1H, H-5), 3.69-3.79 (m, 2H, H-3,4), 3.82 (s, 3H, OCH$_3$), 4.31 (t, 1H, *J* = 5.8 Hz, H-2), 4.51 (m, 1H, OH), 4.92 (d, 1H, *J* = 6.3 Hz, OH), 5.21 (m, 1H, OH), 5.40 (s, 2H, CH$_2$), 5.73 (t, 1H, *J* = 4.5 Hz, OH), 5.80 (t, 1H, *J* = 4.5 Hz, OH), 6.98 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.27 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.49 (d, 1H, *J* = 7.5 Hz, H-1), 9.36 (s, 1H, NH), 10.08 (bs, 1H, NH).
12b	IR: 3482 (OH), 3238 (NH), 1669 (C=O). ^{1}H NMR (DMSO-d_6): 3.30-3.43 (m, 2H, H-6, H-6'), 3.59-3.64 (m, 1H, H-5), 3.70-3.78 (m, 2H, H-3,4), 3.84 (s, 3H, OCH$_3$), 4.30 (t, 1H, *J* = 5.8 Hz, H-2), 4.51 (m, 1H, OH), 4.94 (d, 1H, *J* = 6.3 Hz, OH), 5.22 (m, 1H, OH), 5.40 (s, 2H, CH$_2$), 5.72 (t, 1H, *J* = 4.5 Hz, OH), 5.82 (t, 1H, *J* = 4.5 Hz, OH), 6.97 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.27 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.49 (d, 1H, *J* = 7.5 Hz, H-1), 9.41 (s, 1H, NH), 10.12 (bs, 1H, NH).
12c	IR: 3472 (OH), 3218 (NH), 1666 (C=O). ^{1}H NMR (DMSO-d_6): 3.30-3.43 (m, 2H, H-5, H-5'), 3.59-3.64 (m, 1H, H-4), 3.70-3.78 (m, 2H, H-3,2), 3.81 (s, 3H, OCH$_3$), 4.50 (m, 1H, OH), 4.94 (d, 1H, *J* = 6.3 Hz, OH), 5.19 (m, 1H, OH), 5.38 (s, 2H, CH$_2$), 5.74 (t, 1H, *J* = 4.4 Hz, OH), 6.97 (d, 2H, *J* = 7.6 Hz,Ar-H), 7.30 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.51 (d, 1H, *J* = 7.5 Hz, H-1), 9.39 (s, 1H, NH), 10.08 (bs, 1H, NH).
13a	IR: 3248 (NH), 1736 (C=O), 1662 (C=O). ^{1}H NMR (CDCl$_3$): 1.83, 1.98, 2.05, 2.10, 2.14 (5s, 15H, 5CH$_3$), 3.84 (s, 3H, OCH$_3$), 4.10 (dd, 1H, *J* = 11.2 Hz, *J* = 2.4 Hz, H-6), 4.18 (dd, 1H, *J* = 10.6 Hz, *J* = 2.4 Hz, H-6'), 4.58 (m, 1H, H-5), 5.28 (dd, 1H, *J* = 3.2 Hz, *J* = 6.5 Hz, H-4), 5.38 (t, 1H, *J* = 6.5 Hz, H-3), 5.42 (s, 2H, CH$_2$), 5.68 (dd, 1H, *J* = 3.2 Hz, *J* = 6.2 Hz, H-2), 7.02 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.25 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.51 (d, 1H, *J* = 6.8 Hz, H-1), 9.36 (s, 1H, NH), 10.88 (bs, 1H, NH).
13b	IR: 3242 (NH), 1736 (C=O), 1660 (C=O). ^{1}H NMR(CDCl$_3$): 1.84, 1.98, 2.05, 2.11, 2.15 (5s, 15H, 5CH$_3$), 3.82 (s, 3H, OCH$_3$), 4.11 (dd, 1H, *J* = 11.2 Hz, *J* = 2.4 Hz, H-6), 4.18 (dd, 1H, *J* = 10.6 Hz, *J* = 2.4 Hz, H-6'), 4.55 (m, 1H, H-5), 5.20 (dd, 1H, *J* = 3.2 Hz, *J* = 6.5 Hz, H-4), 5.38 (t, 1H, *J* = 6.5 Hz, H-3), 5.43 (s, 2H, CH$_2$), 5.69 (dd, 1H, *J* = 3.2 Hz, *J* = 6.2 Hz, H-2), 7.04 (d, 2H,*J* = 7.6 Hz, Ar-H), 7.25 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.52 (d, 1H, *J* = 6.2 Hz, H-1), 9.39 (s, 1H, NH), 10.74 (bs, 1H, NH).
13c	IR: 3240 (NH), 1735 (C=O), 1664 (C=O). ^{1}H NMR(CDCl$_3$): 1.86, 2.02, 2.11, 2.14 (4s, 12H, 4CH$_3$), 3.83 (s, 3H, OCH$_3$), 4.09 (dd, 1H, *J* = 11.2 Hz, *J* = 2.4 Hz, H-5), 4.16 (dd, 1H, *J* = 10.6 Hz, *J* = 2.4 Hz, H-5'), 4.82 (m, 1H, H-4), 5.38 (dd, 1H, *J* = 3.8 Hz, *J* = 6.4 Hz, *J* = 6.5 Hz, H-3), 5.40 (s, 2H, CH$_2$), 5.69 (dd, 1H, *J* = 3.2 Hz, *J* = 6.4 Hz, H-2), 7.10 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.27 (d, 2H, *J* = 7.6 Hz, Ar-H), 7.50 (d, 1H, *J* = 6.2 Hz, H-1), 10.02 (s, 1H, NH), 11.02 (bs, 1H, NH).

viously reported procedure (40-43). Compound **2** was used for the preparation of various functionalized triazolopyrimidine derivatives. Reaction of the carboxamide **2** with carbon disulfide in the presence of 10% sodium hydroxide afforded the triazolopyrimidine thione derivative **3** in 77% yield. When compound **2** was allowed to react with triethyl orthoformate, the N-1 substituted triazolopyrimidine derivative **4** was obtained. Reaction of the key compound **2** with 4-chlorobenzaldehyde afforded the 2-chlorophenyl derivative **5**. On the other hand, preparation of the 2-phenyl derivative **6** in 78% yield was started with azide **1** by its reaction with cyanoacetamide and ethyl benzoate. The structures of compounds **3**-**6** were confirmed by their IR, NMR and elemental analyses. Their IR spectra revealed the absence of the charac-

teristic NH_2 absorption bands. The ^{1}H NMR spectra showed, in addition to the aryl and methylene protons, two signals for the NH groups for the thione derivative **3** and one NH signal for compounds **4** and **6**. Furthermore, the ^{1}H NMR spectra of compounds **5** and **6** showed the signals corresponding to protons of the added aryl groups (Scheme 1).

Glycosylation of the pyrimidine derivative **6** was carried out by reaction with the acetylated gluco- and xylopyranosyl bromide derivatives in the presence of potassium hydroxide and produced the corresponding glycosides **7a**,**b**. The ^{1}H-NMR of **7a** showed the anomeric proton of the sugar moiety at δ 5.87 ppm as a doublet, with coupling constants equal to 10.2 Hz indicating the β-orientation of the glycosidic bond. The ^{13}C NMR spectra of **7a** showed a signal at δ 93.8 ppm corresponding to the anomeric C-1, which also confirmed the β-configuration.

Treatment of the acetylated glycosides **7a**,**b** with methanolic ammonia afforded the free hydroxyl glycosides **8a**,**b**, respectively, whose spectral data agree with the assigned structures. Their IR spectra showed characteristic absorption bands for the free hydroxyl groups and their ^{1}H NMR spectra showed the hydroxyl protons signals and revealed the absence of the acetyl-methyl signals which agreed with the assigned structures.

The attachment of thioglycosyl moiety to the triazolopyrimidine ring system was carried out by reaction of the triazolopyrimidyl thione derivative **3** with glucosyl bromide in the presence of potassium hydroxide and produced the corresponding thioglycoside **9**. The formation of glycosylthione derivative rather than the *N*-substituted analog was confirmed on the basis of its spectral data. The ^{1}H NMR spectrum of the produced glycoside showed the anomeric proton of the sugar moiety at δ 5.77 ppm as a doublet, with coupling constants equal to 10.2 Hz indicating the β-orientation of the thioglycosidic bond. The anomeric proton of β-*N*-glycosides having an adjacent C=S was reported to appear at higher chemical shift (δ 6.9–7.2 ppm) due to the anisotropic deshielding effect of the C=S (46-48). The ^{13}C NMR spectra of **9** showed a signal at δ 90.2 ppm corresponding to the anomeric C-1, which also confirmed the β-configuration. The absence of a peak corresponding to the C=S group indicates that the attachment of the sugar has taken place at the sulfur atom and not on the nitrogen atom.

Alkylation of the triazolopyrimidine thione **3** with ethyl iodide in alkaline medium afforded the 2-ethylmercapto derivative **10** in 74% yield. Hydrazinolysis of the produced *S*-ethyl compound gave the required 2-hydrazino derivative **11** in good yield. The ^{1}H-NMR spectrum of compound **10** showed the signals of the ethyl group as triplet and quartet which disappeared in the spectra of the corresponding hydrazine derivative **11**, whereas the NH_2 and NH signals appeared at δ 5.68 and 10.02 ppm, respectively, in addition to signals of the aromatic protons.

When the hydrazine derivative **11** was allowed to react with a number of monosaccharides, namely D-galactose, D-mannose and D-ribose in an aqueous ethanolic solution and with catalytic amount of acetic acid, the corresponding hydrazinyl sugar derivatives were obtained in 75-79% yields. The structures of the produced compounds were confirmed by the analytical and spectral data. The IR spectra of **12a-c** showed the presence of characteristic absorption bands corresponding to the hydroxyl groups in the region 3472–3495 cm^{-1}. The ^{1}H NMR spectra showed the signals of the sugar chain protons at δ 3.30–5.82 ppm, the C-1 methine proton as doublet in the range δ 7.49–7.51 ppm in addition to signals of the methylene and aromatic protons. This high chemical shift value of H-1 indicates the acyclic form of the sugar moiety. The H-1 of sugar moieties in the cyclic form is reported to appear at lower chemical shift values (41). Acetylation of compounds **12a-c** with acetic anhydride in pyridine at room temperature lead to the formation of per-*O*-acetylated derivatives **13a-c**, respectively, in 79-80% yield. The IR spectra showed the presence of absorption bands in the carbonyl frequency region in the range 1731-1736 cm^{-1}. Their ^{1}H NMR spectra revealed the absence of the hydroxyl signals and showed the presence of the acetyl methyl protons at δ 1.83-2.15 ppm in addition to the signals of the sugar chain and aromatic protons.

***In vitro* cytotoxicity activity**

As shown in Table 1, the cytotoxicity of the synthesized compounds was tested using SRB assay in MCF7 and A549 and HCT116 cancer cell lines. For comparison, doxorubicin was used as standard drug, while treatment with DMSO was used as control cancer cells.

Studying the anticancer activity of the new compounds against MCF-7 cell line, revealed that compounds **4**, **7a** and **12a** showed no anticancer activity. Compound **3** exhibited higher potency against MCF-7 cell line with IC_{50} = 2.80 ± 0.33 µg/mL, which is lower than that of doxorubicin (IC_{50} 2.90 ± 0.27 µg/mL). Moreover, the results showed that compounds **2**, **9**, and **10** were found to be potent and selective similar to doxorubicin against MCF-7

cell line with IC_{50} 3.60 ± 0.39, 3.90 ± 4.20 and 4.80 ± 0.55 µg/mL, respectively (Table 1 and Fig. 1).

The cytotoxicity of compounds **2**, **3**, **4**, **6**, **7a**, **9**, **10**, **11** and **12a** was tested against lung cancer cell line A549. For comparison, doxorubicin was also tested. All of the tested compounds except **4**, **7a** and **12a** exhibited anticancer activity and compound **3** (IC_{50} 4.20 ± 0.50 µg/mL) was more potent than doxorubicin (IC_{50} 4.30 ± 0.40 µg/mL), while compound **9**, **10** and **2** were found to be potent near to doxorubicin with IC_{50} 6.50 ± 0.67, 8.00 ± 0.83 and 8.20 ± 0.78 µg/mL respectively. The order of activity was **3**, **10**, **9**, **2**, **11**, **6** in a descending order (Table 1 and Fig. 2).

The results of colon cancer HCT116 cell line revealed that although compounds **4**, **6**, **7a** and **12a** did not exert any activity against the cell, the rest of compounds **2**, **3**, **9**, **10** and **11** had very little anticancer activity compared to doxorubicin (IC_{50} = 4.80 ± 0.50 µg/mL) (Table 1 and Fig. 3).

In conclusion, the tested compounds exert anticarcinogenic activity in breast MCF-7 and lung A549 cancer cell lines through reducing the cell proliferation and resulted in significant growth inhibitory, especially, compounds **2**, **3**, **9** and **10** which revealed promising activity compared to the activity of the commonly used anticancer drug, doxorubicin. The present study reveals that MCF-7 cells are more sensitive to the tested compounds than the other cell lines.

From the above obtained results (Table 1), we can conclude that the attachment of thioxo group at position 2 in the triazolopyrimidine moieties resulted in an enhanced activity. It is obvious that the activity was reduced in other derivatives which do not incorporate such functionality in their structures. Moreover, the attachment of glucosyl moiety to the triazolopyrimidine nucleus through a thioglucosidic linkage increased the activity. This was not the case in nearly similar structures in which the glycosyl moiety is attached to the triazolopyrimidine ring system through C-N linkage. In the present work, the most active compounds were the triazolopyrimidine derivatives **2**, **3**, **9**, and **10** when compared to the reference drug. The difference in activity between the compounds may be attributed to the indicated attachments to the pyrimidine ring of the molecule.

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NATURAL DRUGS

BIOASSAY-GUIDED FRACTIONATION AND ANTIHYPERTENSIVE PROPERTIES OF FRACTIONS AND CRUDE EXTRACTS OF *PERISTROPHE BICALYCULATA* (RETZ) NEES

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Abstract: Hypertension is an important public health issue in both developed and developing countries due to its high incidence and morbidity. This has motivated researchers especially in developing countries to search for strategies for the treatment using different plant parts. The use of the aqueous decoction of the leaves of *Peristrophe bicalyculata* in the treatment of hypertension has been documented. This study was designed to carry out a bioassay-guided isolation of the antihypertensive components of the leaves of *Peristrophe bicalyculata* in L-NAME hypertensive rats, determine the angiotensin-converting enzyme inhibitory activity of the extracts and fractions obtained and identify the constituent(s) present. From our results, L-NAME hypertensive rats given the cold water extract had significantly ($p < 0.05$) lower mean arterial blood pressure (MABP) with longer duration of action than other extracts. Also, the angiotensin-converting enzyme inhibitory activity of the cold water extract was significantly ($p < 0.05$) higher than that of other extracts. From the GC-MS analysis of the most effective fraction (fraction 4), P,P,P-triphenyl-imino(triphenyl)phosphorane and andrographolide 2(3H)-furanone were identified among others. The present work demonstrates the hypotensive effect of the cold water extract of *Peristrophe bicalyculata* on L-NAME hypertensive rats, which further justifies the folkloric application of extracts of the plant in the management as well as treatment of hypertension.

Keywords: angiotensin-converting enzyme, hypertension, *Peristrophe bicalyculata*

Hypertension is a common cardiovascular disease which has become a worldwide problem of epidemic proportions, affecting 15 to 20% of all adults; with ailments such as arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (1). According to the World Health Organization (WHO), about 57 million deaths occurred in 2008, and cardiovascular diseases being the highest recorded, with over 17 million deaths (2). Studies have demonstrated that nearly 90-95% of cases of hypertension are primary and classified as essential hypertension, while the remaining 5-10% is classified as secondary hypertension (3). Substantial evidence demonstrates endothelial dysfunction caused as a result of low nitric oxide (NO) levels and/or increased reactive oxygen species, as a key early event in the development of primary hypertension (4, 5).

Nitric oxide (NO) regulates smooth muscle cell tone, platelet aggregation and adhesion, cell growth, apoptosis, neurotransmission and injury. It also serves as an endogenous vasodilator, platelet inhibitor, antioxidant, and regulator of vascular endothelium by sustaining its anticoagulant and

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antithrombogenic properties (6). In the cardiovascular system, NO is released from the endothelium in response to a physiologic stimulus to activate guanylate cyclase in vascular smooth muscle cells and increase cGMP levels to mediate relaxation (7). The interruption of NO synthesis with several L-arginine analogues such as N^G-nitro-L-arginine methyl ester (L-NAME) has been shown to induce vasoconstriction and arterial hypertension in experimental animals (8) making it a suitable model for studying the antihypertensive effects of new drugs.

Angiotensin converting enzyme (ACE) is a transmembrane zinc metallopeptidase, involved in the regulation of vascular tone by converting angiotensin I (an inactive decapeptide) to angiotensin II (a potent vasoconstrictor). The enzyme also inactivates the vasodilatory nonapeptide bradykinin (9). The ACE inhibitors are used for the treatment of hypertension and prevention of chronic heart failure (10). The effect of ACE inhibitors on the NO/cGMP pathway is not well established (11-13), even though it is well known that the antihypertensive effect of these drugs is attributed to decreased angiotensin II and increased vasodilatory kinins and NO (6). Due to the high incidence and morbidity of hypertension, various drugs and regimes have been advocated for its control. Most of these drugs do not possess complete curative properties; and some demonstrate better efficacy but possess side-effects (14). Recently, attention has been drawn towards herbal sources which are used traditionally as potential therapeutic agents in the prevention and management of cardiovascular diseases (15, 16).

Peristrophe bicalyculata (Retz) Nees is native to warm tropical regions of Africa, in the Sahel part of the region from Mauritania to Niger and Northern Nigeria, India, Burma and Thailand (17). The leaves of the plant have analgesic, antipyretic, anti-inflammatory activities, and antibacterial, fungistatic and bacteriostatic properties (17, 18). Studies have demonstrated the anticancer activity of oils from *Peristrophe bicalyculata* using MCF-7 (human breast tumor) and MDA-MB-468 (human breast tumor) cells (19). The anticancer activity of crude extracts of the plant against Ehrlich ascites carcinoma (EAC) cell lines (20) and human mouth epidermal carcinoma (KB) cells (21) have also been reported. In South-West Nigeria, the plant is used as green manure as well as in the treatment of hypertension and cardiovascular-related diseases (22). The present study was aimed at partially purifying the antihypertensive extract of *Peristrophe bicalyculata*, determine the ACE inhibitory activities of the fractions and identify the antihypertensive component(s)/constituent(s) present.

MATERIALS AND METHODS

Chemicals and reagents

Hippuryl-histidyl-leucine (HHL), angiotensin converting enzyme (ACE), N^G-nitro-L-arginine methyl ester (L-NAME) and captopril were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were of analytical grade.

Preparation of plant material

The leaves of *Peristrophe bicalyculata* (Retz) Nees were harvested at maturity in the month of June, 2010 at Ibadan, Oyo State, Nigeria. The plant was identified and authenticated by the botanist in the herbarium of the Department of Biological Science, Ahmadu Bello University (ABU), Zaria, with a voucher number 2863.

The leaves of the plant were washed clean under running water, air-dried in the laboratory and powdered. The powdered plant (500 g) was defatted in n-hexane before extracting with methanol. The methanol extract was dissolved in distilled water and then partitioned in ethyl acetate and n-butanol using separating funnel, to obtain ethyl acetate fraction of methanol extract, butanol fraction of methanol extract and water fraction of methanol extract. The cold and hot water extracts were obtained by stirring (Harmony Hot Plate Stirrer, Japan) in cold and hot water, respectively, sieved using a muslin cloth and then filtered under suction pressure with Whatman's filter paper (No. 1). All extracts were then concentrated under reduced pressure using a rotary evaporator (Buchi, Switzerland), lyophilized (Christ Alpha 1-2 LD, Germany) and stored at 4^{o}C until needed.

Bioassay-guided fractionation of cold water extract of *Peristrophe bicalyculata*

The cold water extract of *Peristrophe bicalyculata,* was partially purified by column chromatography after thin layer chromatographic separation. The thin layer chromatography was carried out using ethyl acetate : formic acid : methanol (1 : 1 : 3, v/v/v) solvent which gave five separate constituents, visible under UV-spectrum. Column chromatography using silica gel was then run with ethyl acetate (100%), ethyl acetate : formic acid : methanol (15 : 2 : 0.5, v/v/v), ethyl acetate : methanol (1 : 1, v/v), ethyl acetate : methanol (1 : 3, v/v), ethyl acetate : methanol : formic acid (1 : 3 : 1, v/v/v) and methanol

: formic acid (9 : 1, v/v). Elution and fractionation were controlled by TLC and similar fractions were combined.

Determination of angiotensin-converting enzyme inhibitory activity

The assay for ACE inhibitory activity was determined using the Cushman and Cheung (23) method with some modifications on the assay conditions. Briefly, the inhibitor solution (purified extract) was added to 0.1 M potassium phosphate buffer (pH 8.3), which consists of 5 mM hippuryl-histidyl-leucine (HHL), 0.1 M potassium phosphate and 0.3 M NaCl (pH 8.3). Then, the enzyme, ACE was added to the mixture and incubated at 37^{o}C for 30 min. The reaction was terminated by adding 0.25 mL of 1 M HCl, and then 1.5 mL of ethyl acetate was added to extract the hippuric acid formed by the action of ACE. The ethyl acetate was removed by heat evaporation, residual hippuric acid (HA) dissolved in 1 mL of deionized water, and absorbance of the solution was measured at 228 nm to determine the hippuric acid concentration and ACE inhibitory activity. The concentration of ACE inhibitor required to inhibit 50% of the ACE activity under the above assay conditions was defined as IC_{50}.

Experimental design

All experimental protocols were approved and conducted with strict adherence to guidelines and procedures of the Institutional Animal Care and Use Committee of the Natural Product Research and Development Centre, Faculty of Pharmacy, Chiang Mai University, Thailand. One hundred apparently healthy male Sprague-Dawley (SD) rats of about eight weeks' old, weighing between 180 and 230 g were obtained from the National Laboratory Animal Centre, Mahidol University, Salaya, Nakhon Pathom, Thailand, and maintained in an air-conditioned animal house of the Faculty of Pharmacy, Chiang Mai University, Thailand. They were fed normal rat chow and allowed access to clean water *ad libitum*. The rats were allowed to acclimatize for two weeks before the commencement of the experiment.

The experiment was carried out in two phases. In the first phase, rats were divided into 4 groups of 6 rats each: Group 1 (control) - rats were intravenously given normal saline, groups 2, 3 and 4 were intravenously injected with a solution of L-NAME (3 mg/kg) until blood pressure reached maximum. Captopril (5 mg/kg), water fraction of methanol extract (10 mg/kg) and cold water extract (10 mg/kg) were intravenously administered to hypertensive rats in groups 2, 3 and 4, respectively. In the second phase of the experiment; rats were divided into 6 groups of 6 rats each: Group 1 rats were intravenously given normal saline, groups 2, 3, 4, 5 and 6 were intravenously injected with a solution of L-NAME (3 mg/kg) until blood pressure reached maximum. Fractions 1, 2, 3, 4 and 5 obtained after partial purification of the cold water extract, were given to rats in second, third, fourth, fifth and sixth groups, respectively.

Acute toxicity studies

Acute oral toxicity studies of extracts of *Peristrophe bicalyculata* were carried out as described by Lorke (24) with oral administration of increasing doses of the extracts from 10 mg/kg to 5000 mg/kg, while animals were observed for behavioral changes, toxicity and mortality for 24 h.

Blood pressure and heart rate measurement

Rats were fixed on a supine position on a dissecting table and anesthetized by intraperitoneal administration of pentobarbital sodium (40 mg/kg). A longitudinal mid-tracheal incision, approximately 2 cm long was made in order to expose the trachea, the right jugular vein and both common carotid arteries. The trachea was cannulated with a polyethylene tube (2.75 mm diameter) to maintain a free airway, while the right jugular vein was cannulated for the administration of extracts and isotonic saline solution. The systemic blood pressure was recorded from the cannulated left carotid artery, connected to a physiological pressure transducer (Statham P23 AC Strain gauge Tansducer, Laboratories, Inc. Hato Rey, Puerto Rico) and displayed on a Grass 7D Polygraph (Grass Instrument Co., Quincy, Mass, USA). The cannulation of the carotid artery was performed in the same manner as that of the jugular vein, and the polyethylene tube (1 mm diameter) filled with heparin sodium in saline solution was used. Blood pressure and heart rate were monitored until steady base-line levels were obtained. L-NAME at 3 mg/kg was administered *via* venous cannula to induce hypertension. Blood pressure was allowed to stabilize for 30 min before administration of extracts and fractions through the jugular vein cannula. Changes in blood pressure were recognized as difference between the steady state values before and the peak readings after injection (25). Mean arterial blood pressure (MABP) was calculated as the diastolic BP plus one-third of the pulse width (systolic BP - diastolic BP).

GC-MS analysis of the partially purified antihypertensive fraction of *P. bicalyculata*

The gas chromatography and mass spectrometry (GC-MS) analysis was conducted using Shimadzu apparaus with chromatographic system (model GC-2010). The mass analysis apparatus (GC-MS QP2010) was connected to the column RTX-5MS (Restek) 30 m, 0.25 mm internal diameter.

Spectra were generated using the in-built software (GC-MS solution version 2.5 SUI). GC-MS real time and GC-MS post-run analyses were evaluated using three libraries: NIST, Wiley intergrated and a domestic library using a CAS number of EMBRAPA Genetic Resources and Biotechnology. Each spectrum was confirmed by two replicated readings to ensure reproducibility. All peaks were further analyzed for compound identification, based on their similarities with structures available in the libraries.

Statistical analysis

Data obtained were expressed as the mean ± standard error of the mean (mean ± SEM) and analyzed using Statistical Package for the Social Sciences (SPSS Inc., Release 17.0, Chicago, IL., USA). The significance among groups was determined by one way analysis of variance and LSD *post-hoc* test was applied for multiple comparisons. Values of $p < 0.05$ were regarded as statistically significant.

RESULTS

Percentage yield of extracts of *Peristrophe bicalyculata*

The percentage yields of extracts of *Peristrophe bicalyculata* are presented in Table 1. The yields from 50 g powdered plant of the cold and hot water extracts were 9.03 ± 0.44 and 7.79 ± 2.24 g, respectively, while extraction of 500 g with methanol yielded 41.44 ± 5.87 g. Further fractionation of the methanol extract with ethyl acetate, butanol and water yielded 12.28 ± 4.37, 8.61 ± 2.66 and 8.49 ± 3.46 g, respectively.

Acute toxicity

In the acute toxicity study, a single oral administration of all extracts of *Peristrophe bicalyculata* at

Table 1. Yield and percentage yield of extracts of *Peristrophe bicalyculata*.

Extract	Yield (g)	Percentage yield (%)
Cold water (50 g)	9.03 ± 0.44	18.06 ± 0.88
Hot water (50 g)	7.79 ± 2.24	15.58 ± 4.49
Hexane (500 g)	4.61 ± 1.17	1.00 ± 0.23
Methanol (500 g)	41.44 ± 5.87	8.29 ± 1.15
Ethyl acetate of methanol	12.28 ± 4.37	2.46 ± 0.88
Butanol of methanol	8.61 ± 2.66	1.72 ± 0.52
Water of methanol	8.49 ± 3.46	1.70 ± 0.69

Values are the mean ± SEM. Ethyl acetate, butanol and water extracts of methanol extract were obtained from methanol extract by fractionating with ethyl acetate, butanol and water, respectively.

Table 2. Effect of *Peristrophe bicalyculata* on L-NAME-induced hypertension in rats.

Extract	MABP change (%)	HR change (%)	Duration of action (min)
Control	- 2.13 ± 0.03^{c}	1.50 ± 0.02^{f}	-
L-NAME	+ 65.00 ± 5.06^{f}	9.15 ± 2.24^{a}	185.00 ± 51.40
Captopril (5 mg/kg)	- 62.70 ± 5.56^{a}	2.45 ± 1.09^{d}	209.00 ± 12.07^{h}
Water fraction of methanol extract (10 mg/kg)	- 15.67 ± 1.02^{b}	2.67 ± 0.84^{d}	33.33 ± 04.01^{g}
Cold water extract (10 mg/kg)	- 15.16 ± 2.09^{b}	4.33 ± 0.71^{e}	150.00 ± 39.50^{e}

Values are the mean ± SEM. MABP = mean arterial blood pressure; HR = heart rate; $^{a-i}$ = Values with different superscripts in the same column are significantly ($p < 0.05$) different; + : increase in blood pressure; - : decrease in blood pressure, L-NAME = N^{G}-nitro-L-arginine methyl ester.

doses from 10 to 5000 mg/kg did not produce any apparent toxic symptom or mortality after the 24 h observation period.

Effect of *P. bicalyculata* on L-NAME-induced hypertensive rats

The effect of *Peristrophe bicalyculata* on L-NAME-induced hypertensive rats is presented in Table 2. The intravenous administration of L-NAME to normal Wistar rats induced a sustained arterial hypertension which lasted over three hours (185 ± 51.40 min) and significantly ($p < 0.05$) increased MABP by 65 ± 5.06% compared to rats in the control group whose MABP decreased (-2.13 ± 0.03%), but remained constant. Heart rate of hypertensive rats increased significantly ($p < 0.05$) by 9.15 ± 2.24% from 1.50 ± 0.02% in control rats. The MABP was reduced significantly ($p < 0.05$) immediately following intravenous administration of captopril (62.70 ± 5.56%), water fraction of methanol extract (15.67 ± 1.02%) and cold water extract (15.16 ± 2.09%). The reduction in MABP and duration of action (209.00 ± 12.07 min) of captopril in hypertensive rats was significantly ($p < 0.05$) higher than in rats, given water fraction of methanol extract and cold water extract. Although there was no significant ($p > 0.05$) difference in MABP of hypertensive rats given the water fraction of methanol extract and cold water extract, the duration of action of the cold water extract (150.00 ± 39.50 min) in hypertensive rats was significantly higher ($p < 0.05$) than in those given water fraction of methanol extract (33.33 ± 04.01 min).

Table 3. Fractions and yields of cold water extract of *Peristrophe bicalyculata* obtained by column chromatographic separation.

Solvents	Fractions	Yield (g)	New fractions
Ethyl acetate (100%)	Fraction 1	0.0062	1
Ethyl acetate : formic acid : methanol (15 : 2 : 0.5)	Fraction 2a	0.0042	(Fractions 1 and 2a)
	Fraction 2b	0.2127	2 (Fraction 2b)
	Fraction 2c	1.3438	3 (Fraction 2c)
Ethyl acetate : methanol (1 : 1)	Fraction 3	0.2043	4
Ethyl acetate : methanol (1 : 3)	Fraction 4	0.0388	(Fractions 3 and 4)
Ethyl acetate : methanol : formic acid (1 : 3: 1) Methanol : formic acid (9 : 1)	Fraction 5a	0.6499	(Fractions 5a, 5b,5c and 6)
	Fraction 5b	0.1936	
	Fraction 5c	0.0516	
	Fraction 6	0.0077	
Total		**2.7128**	

Table 4. Effect of fractions of cold water extract of P. *bicalyculata* on L-NAME hypertensive rats.

Groups	MABP change (%)	HR change (%)	Duration of action (min)
Control	1.21 ± 0.06^{a}	1.67 ± 0.10^{h}	-
L-NAME (3 mg/kg)	$+60.00 \pm 9.02^{b}$	12.15 ± 2.62^{i}	161.02 ± 18.09
Captopril (5 mg/kg)	-40.14 ± 9.08^{c}	3.13 ± 1.06^{m}	$182.00 + 30.21^{s}$
Fraction 1 (0.03 mg/kg)	$+13.17 \pm 2.43^{d}$	$9.17 \pm 0.65^{k,i}$	120.00 ± 80.50^{x}
Fraction 2 (0.71 mg/kg)	-21.00 ± 2.94^{e}	4.67 ± 0.76^{m}	102.83 ± 43.32^{y}
Fraction 3 (4.48 mg/kg)	-12.67 ± 1.60^{f}	$6.00 \pm 2.62^{k,m}$	116.67 ± 37.16^{y}
Fraction 4 (0.81 mg/kg)	-29.33 ± 2.26^{g}	4.33 ± 0.80^{m}	69.17 ± 13.57^{z}
Fraction 5 (3.00 mg/kg)	-25.17 ± 1.11^{e}	2.33 ± 1.11^{m}	65.00 ± 3.16^{w}

Values are the mean ± SEM. MABP = mean arterial pressure; HR = heart rate; $^{a-z}$ = Values with different superscripts in the same column are significantly ($p < 0.05$) different; + : increase in blood pressure; - : decrease in blood pressure
L-NAME = N^{G}-nitro-L-arginine methyl ester.

Table 5. Angiotensin-converting enzyme inhibitory effect of cold water extract of *Peristrophe bicalyculata*.

Samples	IC_{50} (µg/mL)
Captopril	2.38 ± 0.19[a]
Fraction 1	24.98 ± 1.73[b]
Fraction 2	27.23 ± 1.60[b]
Fraction 3	15.86 ± 1.78[c]
Fraction 4	9.40 ± 1.58[d]
Fraction 5	13.85 ± 0.78[c]

Values are the mean ± SEM. [a-e] = Values with different superscript letters are significantly different ($p < 0.05$).

Bioassay-guided fractionation of cold water extract of *Peristrophe bicalyculata*

From the results (Table 3), 10 different fractions were obtained; with the ethyl acetate (100%), ethyl acetate : methanol (1 : 1, v/v), ethyl acetate : methanol (1 : 3, v/v) and methanol : formic acid (9 : 1, v/v) solvents, giving one fraction each (fractions 1, 3, 4 and 6, respectively); while ethyl acetate : formic acid : methanol (15 : 2 : 0.5, v/v/v) and ethyl acetate : methanol : formic acid (1 : 3 : 1, v/v/v) yielded 3 fractions each: fractions 2a, 2b, 2c and 5a, 5b and 5c, respectively. The yield from fraction 2c was the highest (1.3738 g), which was about 50% of the extract used; and the least was fraction 2a (0.0042 g). The fractions were reduced to five after TLC separation, based on their spots color, retention factor and physical characteristics. These fractions were then administered to hypertensive rats to determine the most active.

Effect of fractions of cold water extract of *P. bicalyculata* on hypertensive rats

From the results (Table 4), the percentage MABP of L-NAME-induced hypertensive rats increased significantly ($p < 0.05$) by 60.00 ± 9.02% and persisted for over 2.5 h (161.02 ± 18.09 min), while their heart rates also increased by 12.15 ± 2.62%. Following intravenous administration of captopril, and fractions 2, 3, 4 and 5, the MABP was significantly ($p < 0.05$) reduced by 40.14 ± 9.08%, 21.00 ± 2.94%, 12.67 ± 1.60%, 29.33 ± 2.26% and 25.17 ± 1.11%, respectively. The intravenous administration of captopril in rats significantly ($p < 0.05$) reduced MABP with a longer duration of action (182.00 + 30.21 min) than in those given other fractions. Fraction 4 at 0.81 mg/kg reduced MABP of hypertensive rats by 29.33 ± 2.26% for over one hour, which was significantly ($p < 0.05$) higher than reduction percentages induced by fractions 2 (21.00 ± 2.94%), 3 (12.67 ± 1.60%) and 5 (25.17 ± 1.11%). Conversely, fraction 1 increased blood pressure of hypertensive rats by 13.17 ± 2.43%.

The percentage decrease in heart rate of hypertensive rats given fractions 4 (4.33 ± 0.80%) and 5 (2.33 ± 1.11%) did not differ significantly ($p > 0.05$) from each other.

Angiotensin-converting enzyme inhibitory activity of extracts and fractions of *Peristrophe bicalyculata*

Results of the ACE inhibitory activity of the fractions of *Peristrophe bicalyculata* are presented in Table 5. As shown, captopril inhibited 50% of the ACE activity (IC_{50}) at a concentration (2.38 ± 0.19 µg/mL) significantly lower ($p < 0.05$) than that recorded for any other fraction. The ACE inhibitory activity of fraction 4 (9.40 ± 1.58 µg/mL) was significantly ($p < 0.05$) lower than all other fractions, while, fractions 1 and 2 inhibited the enzyme at the highest concentrations (24.98 ± 1.73 and 27.23 ± 1.60 µg/mL, respectively).

Gas chromatography and mass spectrometry analysis of the partially purified antihypertensive fraction of *Peristrophe bicalyculata*

The results of the GC-MS analysis identified the various compounds present in the partially-purified fraction (Table 6). Figure 1 shows the gas chromatogram with 5 distinct peaks identified by the GC-MS.

The major compound identified by GC-MS in the partially-purified antihypertensive fraction of *Peristrophe bicalyculata* was P,P,P-triphenylimino(triphenyl)phosphorane with retention time (RT) 47.28 min and molecular weight (m.w.) of 278 g. Other compounds present are propanoic acid (RT 3.41 min; m.w. 102 g), 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (RT 11.71 min; m.w. 144 g), 1,1,1,5,7,7,7-heptamethyl-3,3-bis(trimethylsiloxy)-tetrasiloxane (RT 42.79 min; m.w. 444 g) and andrographolide 2(3H)-furanone (RT 58.62 min; m.w. 350 g).

DISCUSSION

The antihypertensive and ACE inhibitory activities of the cold water extract and water fraction of methanol extract were investigated based on the result of previous study (26). In the present study, the effect of the extract on L-NAME-induced hypertensive rats was investigated. This is because it is well established that the alteration of the nitric oxide

Table 6. Components identified in antihypertensive fraction of cold water extract of *Peristrophe bicalyculata* by GC-MS.

Name of compound	Retention time (min)	Molecular formula	Molecular weight (g)
Propanoic acid	3.41	$C_5H_{10}O_2$	102
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	11.71	$C_6H_8O_4$	144
1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	42.79	$C_{13}H_{40}O_5Si_6$	444
Pentadecanal	45.40	$C_{15}H_{30}O$	226
P,P,P-triphenyl-imino(triphenyl)phosphorane	47.28	$C_{18}H_{16}NP$	277
Diazoprogesterone	49.85	$C_{21}H_{30}N_4$	338
5-Ethyl-2-nonanol	51.07	$C_{11}H_{24}O$	172
Arachidonic acid trimethylsilyl ester	56.31	$C_{23}H_{40}O_2Si$	376
Andrographolide 2(3H)-furanone	58.62	$C_{20}H_{30}O_5$	350
Pseduosarsasapogenin-5,20-dien methyl ether	61.40	$C_{28}H_{44}O_3$	428

The compounds presented in the Table are those which matched similar compounds in the NIST, Wiley intergrated and a domestic library using a CAS number of EMBRAPA Genetic Resources and Biotechnology and which contained the molecular ion of the matching compound.

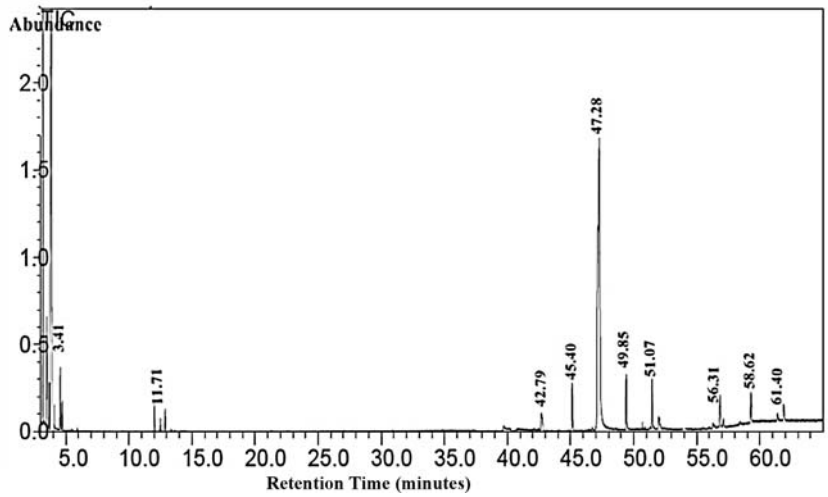

Figure 1. Gas chromatogram of partially-purified antihypertensive fraction of *Peristrophe bicalyculata*

system plays an important role in the development or maintenance of clinical hypertension (27). This model of hypertension also mimics essential hypertension, which affects 90-95% of hypertensive individuals as it is usually caused by endothelial dysfunction due to the inhibition of NO (28).

The cold water extract of *Peristrophe bicalyculata* was found to be the most effective in reducing MABP of hypertensive rats. This significant ($p < 0.05$) reduction in MABP observed in L-NAME hypertensive rats after acute intravenous administration of the cold water extract which lasted over 2 h validates the claim that the aqueous extract of the plant possesses antihypertensive activity (26). Although the percentage reduction of MABP by the cold water extract (15.16 ± 2.09%) and water fraction of methanol extract (15.67 ± 1.02%) was not significantly different ($p > 0.05$), the duration of action of the cold water extract was significantly ($p < 0.05$) higher, hence our choice of further purifying the cold water extract. The consistent and normal MABP in rats within the control group is an evidence that the rats were healthy and not hypertensive, while acute administration of L-NAME to the rats significantly increased MABP. It is well documented that acute intravenous administration of L-NAME results in sustained hypertension attributed to the inhibition of nitric oxide synthase, known to

inhibit the synthesis of nitric oxide from L-arginine, thereby causing severe and progressive arterial hypertension (8, 29).

It has previously been reported that the cold water extract of *Peristrophe bicalyculata* possesses significant ACE inhibitory activity (22) compared to other extracts of the plant. In the present study, partial purification of the cold water extract yielded five fractions; with fraction 4 inhibiting ACE at the lowest concentration compared to other fractions; and thus, considered the most active inhibitor. Fraction 4 also reduced MABP significantly ($p < 0.05$) at a lower concentration (0.81 mg/kg) compared to others (Table 4). Thus, we may suggest that the antihypertensive effect of *Peristrophe bicalyculata* may be due in part to its ability to prevent the conversion of angiotensin I to angiotensin II by inhibiting ACE in the renin-angiotensin system (RAS). This is consistent with studies by Jimenez-Ferrer et al. (30) that antihypertensive plants with good ACE inhibitory activity exert their effect by inhibiting the conversion of angiotensin I to angiotensin II. However, several studies have demonstrated that the generalized vasoconstriction characteristic of chronic nitric oxide synthase (NOS) inhibition model is maintained by an interaction between the sympathetic nervous system and the RAS, (31, 32), whereas, in the acute NOS inhibition model, the RAS may not be involved (33). Thus, it is assumed that ACE inhibitors may alleviate acute hypertension by synthesis of vasodilatory substances such as NO, prostacyclin, and endothelium-dependent hyperpolarizing factor (EDHF) (11, 34, 35). Thus, we may postulate that the mechanism underlying the acute antihypertensive effect of the cold water extract and partially purified fraction (fraction 4) of *Peristrophe bicalyculata*, which in the present study were found to be potent ACE inhibitors, may be linked to the synthesis of vasodilatory substances by bradykinin, as it has been shown that ACE inhibition enhances the vasodilator effects of bradykinin and that blockade of the bradykinin receptor attenuates the hypotensive action of ACE (36).

The potent antioxidant activity of the plant (37) may have played a significant role in alleviating hypertension (30, 38), as phenols have been reported to enhance vascular NO activities by inducing NO production through NOS expression or by protecting NO against destruction, while flavonoids are known to possess cardioprotective and antihypertensive effects by specifically targeting cardiovascular ionic channels and playing important roles in vascular tone regulation (39). Furthermore, flavonoids from different plant sources have been found to inhibit ACE (40, 41). Flavones of Roxb., apigenin and luteolin have demonstrated a dose-dependent enzyme inhibition (10). Thus, the antioxidant effects of the plant may have contributed in reducing blood pressure by synthesizing vasodilatory substances and scavenging free radicals, known to cause oxidative stress.

The significant ($p < 0.05$) reduction in heart rate of hypertensive rats given captopril (Tables 3 and 5) corroborates studies showing that ACE inhibitors such as captopril reduces heart rate (42, 43), while, others (44, 45) have shown that it is not affected by captopril. Captopril is a potent vasodilator, and several vasodilators have been shown to increase heart rate due to increased cardiac function and oxygen consumption. However, it has been reported that captopril decreases blood pressure without increasing heart rate due to its ability to increase vagal tone as a result of angiotensin II inhibition (46, 47). To the best of our knowledge, the effect of *Peristrophe bicalyculata* on heart rate has not been reported, but our results contradict the findings of Zhang et al. (48), demonstrating that *Andrographis paniculata*, a plant with antihypertensive properties, belonging to the same family as *Peristrophe bicalyculata*, has no effect on heart rate. However, more experiments need to be done especially on aortic vasorelaxation to determine the exact mechanism of heart rate reduction.

The cold water extract of *Peristrophe bicalyculata* did not exhibit acute toxicity up to the maximum dose of 5000 mg/kg, suggesting that it is practically non-toxic, and may be a good source of pharmacological material (24).

P,P,P-triphenyl-imino(triphenyl)phosphorane was found to be the most abundant compound present in the partially purified antihypertensive fraction of *Peristrophe bicalyculata,* but may not necessarily be responsible for the antihypertensive activity of the plant, as there is a need to isolate and test all compounds individually before making a logical conclusion. This compound is a derivative of immunophosphoranes, known to be important reagents in synthetic organic chemistry for the synthesis of naturally occurring products, compounds with biological and pharmacological activity (49). It is also possible that the compound may have improved the biological function of the antihypertensive compound as immunophosphoranes are used for the modification of cell surfaces, protein engineering, proteomic studies, labeling of nucleic acids and as tools for bioconjugation (50), or may have improved the antioxidant or antihypertensive activity of the plant (51).

In conclusion, this study demonstrates the acute antihypertensive properties of *Peristrophe bicalyculata* in NO-deficient hypertensive rats. The acute antihypertensive effects might be due to the synthesis of vasodilatory substances by bradykinin as well as the ability of the plant to act as an effective antioxidant. The data obtained also provide useful leads in the development of an effective anti-hypertensive drug from *Peristrophe bicalyculata*. Isolation and purification of the antihypertensive component await further study.

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PRELIMIARY PHYTOCHEMICAL ANALYSIS AND ANTIFUNGAL ACTIVITIES OF CRUDE EXTRACTS OF *ZALEYA PENTANDRA* AND *CORCHORUS DEPRESSUS* LINN.

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Abstract: *Zaleya pentandra* (*Zp*) and *Corchorus depressus* Linn. *(Cd)* have been considered as herbs with potential therapeutic benefits. *Zp* and *Cd* belong to the important family Aizoaceae and Tiliaceae, respectively.The extractions were carried out successively with methanol and dichloromethane at room temperature for 24 h. Preliminary phytochemical screening of *Zp* and *Cd* revealed the presence of steroids, alkaloids, saponins, and anthraquinones. The methanolic and dichloromethane extracts of selected plants were subjected to examination of antifungal activity by using agar tube dilution. The extracts were tested against different fungi such as *A. niger, A. flavus, F. solani, A. fumigatus* and *Mucor.* The dichloromethane extract of aerial parts of *Cd* showed high antifungal activity against *A. niger as* compared to all other tested extracts.

Keywords: *Zaleya pentandra*, *Corchorus depressus* Linn. Aizoaceae, Tiliaceae

The page of human history reveals that human beings irrespective of ethnicity or belonging to any civilization have been utilizing herbs as medicine for the purpose of healthcare (1). Medicinal plants are of great value in the mitigation and cure of diseases. Over the years, scientific research has advanced our knowledge of medicinal plants and new drugs (2).

In the Western world, as people are becoming aware of the potency and side effects of synthetic drugs, there is an increasing interest in plant-based medications. The future development of the pharmacognostic analysis of herbal drugs is largely dependent upon reliable methodologies for correct identification, standardization and quality assurance of herbal drugs (3).

In plants, defence system - natural active compounds perform a major role. These compounds are also known for the physiological action on human body. Primary metabolites in plants are: amino acids, sugars, proteins and chlorophyll. The secondary metabolites, like flavonoids, saponins, are very important therapeutic agents and are becoming very important part of the integrative health care system as alternative medicines. Infectious diseases - the main threats to the public health, are still due to the bacteria, viruses and fungi. In developing nations, this factor is prominent due to unavailability of the medicines. The second reason for the greater impact is drug resistance of microorganisms. Due to the factors mentioned there is the need for the discovery of new antimicrobial compounds (4).

Zaleya pentandra is widely distributed prostrate and branched herb. A genus of about 6 species found in Africa, Asia, Australia has only one specie, *Zp*, found in Pakistan. The plant has been used as astringent while locally used against malaria and snake bite. Previously, it was reported against influenza and phlegmatic cough (5). This plant has been reported to have antifungal, antiulcer, antisecretory, antivenom activity (6) and as cytoprotective (6, 7). Phytochemical evaluation of *Zp* showed the presence of phytosterolins (8). Another medicinal herb *Corchorus depressus* Linn. (*Cd*), commonly known as Boa-phalee, belongs to the family Tiliaceae. This family has 50 genera and 450 species which are distributed in tropical and temperate regions of mainly South Asia and South America. In Pakistan, about four genera and 24 species are found; *Cd* is included among cultivated species. *Cd*

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is used as a traditional medicine for the ailment of aches, dysentery, enteritis, fever and tumors (9). The genus Corchorus has been reported for anticancer and cardiac activity in Indian medicinal system. The infusion of leaves is a demulcent, laxative, carminative, stimulant, appetizer and tonic. *Cd* has been reported as analgesic and antipyretic (10). Literature survey of this plant revealed phytochemical constituents: triterpenoids, sterols (11) and flavonoids (12) reported from chloroform extract of *Cd. pentandraone,* a novel compound isolated from *Zp* (13). Plant is reported as antimalarial (14), antifungal and antibacterial (11, 15). Keeping in view above mentioned literature, it is obvious that both medicinal plants have great therapeutic potential and are available in ample amount by cultivation. The objective of this study was to validate the *Cd* and *Zp* plants against different strains of fungi by using different parts of plants in different types of solvents and preliminary phytochemical analysis of these emerging therapeutic agents .

MATERIALS AND METHODS

The plants *Cd* and *Zp* were collected from Peruwal (District Khanewal). The plants were identified by Prof. Dr. Altaf Ahmad Dasti, Plant Taxonomist, Institute of Pure and Applied Biology, Bahauddin Zakariya University Multan, Pakistan, where their voucher specimens fl. p. 472/4 for *Cd* and fl. p. 235/5 for *Zp* were deposited.

Extraction

The shade-dried aerial parts and root part of *Cd* (1000 g) and aerial parts of *Zp* (1000 g) were subjected to extraction successively with dichloromethane and methanol (3 × 6 L), respectively, at room temperature with occasional shaking for 24 h. Extracts were concentrated by Rotavapor-R200 at 35°C. The methanolic and dichloromethane extracts of selected plants were collected in separate sample bottles labelled with different codes. The results are depicted in Table 1.

Antifungal assay

To study antifungal activity, we followed agar tube dilution method. It was according to the protocol of Duraipandiyan and Ignacimuthu (16) to determine this activity of plant extracts. In order to grow fungus we used potato dextrose agar (PDA -Merck) media and it was used for inoculum preparations. It was composed of agar 15 g/L, dextrose 20 g/L and potato infusion 4 g/L, approximately. For media preparation for fungus, we dissolved 9.75 g of potato dextrose agar (PDA) in 250 mL of distilled water and then autoclaved the PDA media. The samples (15 mg/mL) were prepared and got concentration (250 µL/mL) for antifungal assay. Nystatin 100,000 units/mL was used as positive control while DMSO was utilized as negative control.

Then, PDA media were dispensed as 4 mL volumes into autoclaved cotton plugged and screw capped test tubes and these were marked to 10 cm mark. These test tubes were cooled up to 50°C and these media were seeded with 67 µL of sample from stock solution of plant extracts with the help of pipette. In this way we got a final concentration of 250 µg/mL. Test tubes were then placed in slanting position and allowed to solidify at room temperature. A 4 mm part of fungus inoculum was subjected to inoculation in the tubes with solidified media and the test sample. Positive test tubes and negative test tubes having nystatin and DMSO, respectively, were inoculated with fungus. After that these test tubes were put in incubator at 30°C for 48 h. Then, linear growth of fungus was calculated in the slant. As far as growth inhibition was required, it was measured with reference to negative control of DMSO. The experiment was performed in triplicate for each fungus and sample. The percentage inhibi-

Table 1. Results of the extraction of the plants *Zaleya pentandra* and *Corchorus depressus*.

Plant name	Part used	Solvent	Weight of extract (g)	Abbreviation for the extracts
Corchorus depressus	Aerial parts (1000 g)	Dichloromethane	39.85	CDD
		Methanol	7.95	CDAM
Corchorus depressus	Roots (1000 g)	Dichloromethane	9.4	CDRD
		Methanol	17.84	CDRM
Zaleya pentandra	Aerial parts (1000 g)	Dichloromethane	33	ZPAD
		Methanol	35	ZPAM

tion of fungal growth was calculated by applying the formula given as follows:

$$\% \text{ inhibition of fungal growth} = \frac{10 - \text{Linear growth in test (cm)}}{\text{Linear growth in control (cm)}} \times 100$$

Detection of various classes of secondary metabolites

Phytochemical studies were carried out for the detection of alkaloids, glycosides and saponins in different parts of the plant *Cd* and Zp. These tests were performed as preliminary basis to identify various classes of metabolites.

Detection of alkaloids

Ten grams of the ground plant material was boiled with 10 mL of acidified water in test tube for 1 min, cooled, and then allowed the debris to settle. The supernatant liquid was filtered into another test tube, 1 mL of this filtrate was taken and 3 drops of Dragendorff reagent were added, there was no precipitate. The remainder of the filtrate was made alkaline by addition of dilute ammonia solution. Five milliliters of chloroform was added to the solution in separating funnel and two layers were observed. The lower chloroform layer was separated out into another test tube. Chloroform layer was extracted by addition of 10 mL of acetic acid and then chloroform was discarded. Then, the extracts were divided into three portions, to one portion few drops of Dragendorff reagent and to second few drops of Mayer's reagent were added. Turbidity or precipitate was compared with the third untreated control portion (16).

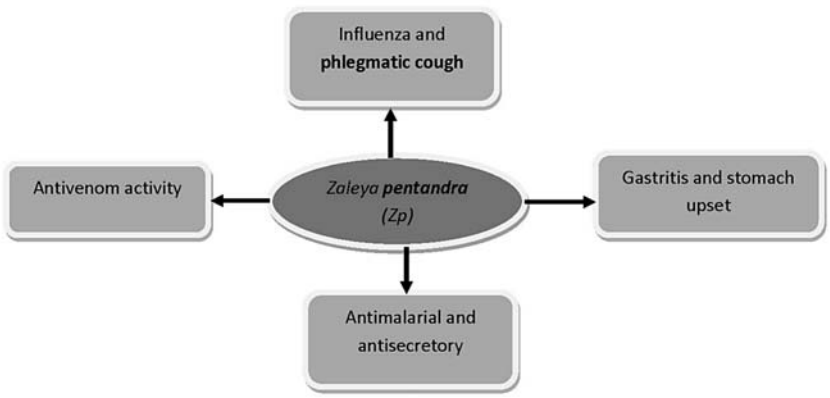

Figure 1. Reported pharmacological activities of *Zaleya pentandra*

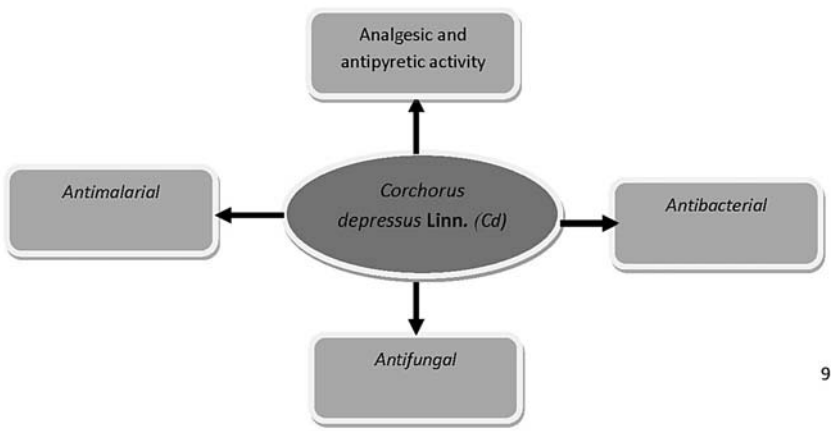

Figure 2. Reported pharmacological activities of *Corchorus depressus* Linn.

Detection of anthraquinone glycosides

Borntragers test

Two grams of powdered plant material were taken and extracted with 10 mL of hot water for 5 min, allowed to cool and filtered; the filtrate was extracted with 10 mL of carbon tetrachloride. Then, carbon tetrachloride layer was taken off, washed with 5 mL of water and then 5 mL dilute ammonia solution was added. No free anthraquinones were revealed as there was no appearance of pink to cherry red color in the ammoniacal layer.

Two grams of second sample of the plant material was extracted with 10 mL of ferric chloride solution and 5 mL of hydrochloric acid by heating it on water bath for 10 min and then filtered. The filtrate was cooled and treated as above (17).

Detection of cardioactive glycosides

Keller Kiliani test

One gram of ground plant material was taken in a test tube and 10 mL of 70% alcohol was added. It was then boiled for 2 min and filtered. The filtrate was diluted twice of its volume with water and then 1 mL of strong lead acetate solution was added. This treatment leads to the precipitation of chlorophyll and other pigments, which were then filtered off. The filtrate was extracted with an equal volume of chloroform. Chloroform layer was separated out and evaporated to dryness in a dish over a water bath. The residue was dissolved in 3 mL of 3.5% ferric chloride in glacial acetic acid and was transferred to test tube after leaving for 1 min. Sulfuric acid (1.5 mL) was then added, which formed a separate layer at the bottom. Cardioactive glycosides were revealed by the appearance of brown color at interface (due to deoxysugar) on standing and appearance of pale green color in the upper layer (due to the steroidal nucleus) (17).

Detection of saponins glycosides

For the detection of saponin, standard procedure for identification of plant constituents was adopted from previously reported methodology (18). Three grams of each extract was extracted with 300 mL of hot distilled water. After extraction, extracts were stored at 4^{o}C. Approximately 5 mL of each extract was diluted with equal volume of distilled water, put in a beaker and shaken vigorously for 2 min. For this test 0.5 g of powdered drug were taken in test tube and shaken with water. Persistent foam indicated the presence of saponins. Persistent foam formation, with the addition of olive oil and lasting for minimum 15 min confirmed the presence of saponins

RESULTS AND DISCUSSION

Medicinal plants have been studied extensively from pharmacological and pharmaceutical chemistry point of view to isolate and identify novel therapeutic compounds. As shown in Figures 1 and 2, substantial studies are required to endorse these medicinal plants as therapeutic remedy in different ailments. An effort was made to evaluate antifungal activities and preliminary phytochemical analysis of two medicinal plants of Pakistan *Cd* and *Zp*. Five strains of fungi *A. niger, A. flavus, F. solani, A. fumigates and Mucor* were selected to evaluate the antifungal activity of different extracts of these plants. Different parts of both plants were selected and different solvents were used to find out most active fraction of plants.

The shade-dried aerial parts and root part of *Cd* and aerial parts of *Zp* were subjected to extraction successively with dichloromethane and methanol at room temperature with occasional shaking for 24 h. The extracts were concentrated under reduced pressure. Then, they were collected in sample bottles labelled with different codes.

Phytochemical screening of the selected plants revealed the presence of alkaloids, steroids, anthraquinones and saponins (Table 2). The methanolic and dichloromethane extracts of selected plants were subjected to estimation of antifungal

Table 2. Results of phytochemical screening of *Corchorus depressus* and *Zaleya pentandra*.

Name of plant	Alkaloids	Anthraquinones	Cardiac glycosides	Saponins
Corchorus depressus	+	-	+	+
Corchorus depressus (root part)	+	-	+	+
Zaleya pentandra	+	-	+	+

+ = present; - = absent

Table 3. Results of antifungal activities of dichloromethane and methanol extracts of *Corchorus depressus* and *Zaleya pentandra* (Growth inhibition of fungal strains after 48 h).

Plant extract	Percentage inhibition of fungal growth of				
	A. niger	*A. flavus*	*F. solani*	*A. fumigates*	*Mucor*
ZPAD	89.70	0	49.07	0	0
ZPAM	47.02	0	34.13	6.48	88.47
CDRD	43.39	87.82	64.88	19.70	49.13
CDRM	87.31	77.59	55.31	12.33	55.07
CDAM	61.20	89.24	24.78	6.94	77.70
CDAD	90.08	51.24	29.86	3.32	0
P Control	117.45	113.88	102.68	109.42	106.44
N Control	27.61	21.70	12.35	19.82	14.64

P Control = Positive control (standard); N Control = Negative control (standard).

Table 4. Results of antifungal activities of dichloromethane and methanol extracts of *Corchorus depressus* and *Zaleya pentandra* (Growth inhibition of fungal strains after 72 h).

Plant extract	Percentage inhibition of fungal growth of				
	A. niger	*A. flavus*	*F. solani*	*A. fumigates*	*Mucor*
ZPD	59.01	0	27.58	0	0
ZPAM	28.96	0	1.26	0	64.45
CDRD	35.53	67.28	30.98	0	24.43
CDRM	59.12	51.31	18.46	0	22.83
CDAM	52.63	65.49	0	0	55.29
CDAD	73.04	27.70	0	0	0
P Control	102.16	96.74	89.03	97.15	96.39
N Control	14.62	5.26	0	9.63	5.88

P Control = Positive control (standard); N Control = Negative control (standard).

activity by using agar tube dilution. The antifungal activity of the plant extracts were determined against the five different fungi named above (Tables 3, 4).The dichloromethane extract of *Zp* and methanolic extract of *Cd* of root part and dichloromethane extract of *Cd* of aerial part actively inhibited the growth of fungus *A. niger* and the maximum growth was 3.15 cm with these extracts. *A. flavus* showed maximum growth inhibition with dichloromethane and methanolic extract of root and aerial parts of *Cd*. The growth with methanolic extract of root part of *Cd* was 3.63 cm. In the presence of plant extract, dichloromethane extract of root part of *Cd* against *F. solani* showed little growth (4.22 cm.) The growth of *Mucor* with methanolic extract of *Zp* and methanolic extract of *Cd* weres 2.3 cm and 3.2 cm, respectively. The plant extracts also showed growth inhibition after 72 h of incubation .The dichloromethane extract of *Zp*, methanolic root part and dichloromethane extract of *Cd* showed 80% or more growth inhibition against *A. niger.* It has been reported that *A. niger* is the cause of pulmonary infection (19).

Similarly, methanolic extract of aerial part and dichloromethane extract of root part of *Cd* showed more than 80% growth inhibition against *A. flavus*. It has been reported that *A. flavus* cause alatoxicosis (20). The methanolic extract of *Zp* showed more than 80% growth inhibition against *Mucor* after 48 h. This extract showed more than 55% growth inhibition after 72 h. Interestingly, in the case of *A. fumigatus,* no significant growth inhibition was observed. The methanolic extract of root parts showed 55% and dichloromethane extract of *Cd* showed 64% growth inhibition respectively, against fungus *F. solani*.

CONCLUSION

Among all the solvents and plant parts, methanolic extract of aerial part of *Zp* showed the highest percentage of growth inhibition against all strains of fungi except *A. flavus,* whereas methanolic extract of aerial parts of *Cd* showed greater percentage of growth inhibition against all strains of fungi. Both plants have been proved as potential antifungal agents.

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RAPESEED PHOSPHATIDYLCHOLINE HYDROLYSIS TO PHOSPHATIDIC ACID USING PLANT EXTRACTS WITH PHOPSPHOLIPASE D

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Abstract: Phosphatidic acid (PA) has a crucial role in cell membrane structure and function. For that reason it has a possible application in the treatment of some health disorders in humans, can be used as a natural and non toxic emulsifier and the component of drug carriers in pharmaceuticals and cosmetics as well as a component for synthesis of some new phospholipids. PA is short-lived in the cell and is difficult to extract directly from the biological material. PA may be easily prepared by hydrolysis of phospholipids, especially phosphatidylcholine (PC), using cabbage phospholipase D (PLD). Hydrolytic activity of purified by us PLD extracts from cabbage towards rapeseed phosphatidylcholine (RPC) was investigated. Hydrolysis was carried out in the biphasic system (water/diethyl ether) at pH 6,5 and temp 30˚C. Influence of enzymatic extracts from three cabbage varieties, reaction time, Ca^{2+} concentration and enzyme extracts/PC ratio, on activity towards RPC resulting in rapeseed phosphatidic acid (RPA) formation were examined. Our study shows that the PLD extracts from savoy cabbage (PLD_{sc}), white cabbage (PLD_{wc}) and brussels sprouts (PLD_{bs}) used in experiments exhibit hydrolytic activity towards RPC resulting in rapeseed RPA with different yield. The highest activity towards RPC shows PLD extract from PLD_{sc} with the RPC conversion degree to RPA (90%) was observed at 120 mM Ca^{2+} concentration, reaction time 60 min and ratio of PLD extract to RPC 6 : 1 (w/w). Our study shows that purified by us PLD_{sc} extracts exhibit hydrolytic activity towards RPC giving new RPA with satisfying conversion degree for use in pharmacy, cosmetics and as a standard in analytical chemistry.

Keywords: rapeseed phosphatidylcholine , phospholipase D from cabbage, rapeseed phosphatidic acid, phospholipid hydrolysis

Using phospholipases as biocatalysts is an interesting concept in the modification of oxygen and heat sensitive plant phospholipids. Contrary to the chemical modification, enzymatic methods are more safe and distinguished, especially for the products used in pharmaceuticals, cosmetics and food. Enzymes are selective and stereospecific catalysts, so the hydrolysis by them can be carried out under mild conditions, eliminating the use of toxic and harmful solvents, which often are present in a form of residue, when the chemical modifications were used (1). Phosphatidic acid (PA) was first reported as a component of cabbage leaf cytoplasm (2) and is an acid, negative charged phospholipid, the structural component of many cell membranes. PA is generated in animal and plant cells mainly *via* hydrolysis of phospholipids, in particular phosphatidylcholine (PC) by phospholipase D (3-5). In cells PA acts as biosynthesis precursor of many other phospholipids and intracellular lipid second messenger. This presents new opportunities for development of therapeutic approaches relevant to reproduction, metabolism and neurodegenerative disease (6). Rutenberg reported that combination of phosphatidylserine and PA has been shown to have anti-depressant, stress suppressor and mood improver component (7). PA has also been examined as a dietary supplement which enhanced strength, muscle thickness and lean tissue during training program. The ability to augment muscle strength is important for various population groups (elderly people, athletes) (8). Orally administrated PA presents opportunity to prevent nonsteroidal anti-inflammatory drug induced gastric ulcer, and can be considered as a potential antiulcer phospholipid (9, 10). It was also found that formulation composed of PA and α-lactoglobulin is

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inhibitory on bitterness and may be used for practical purposes to inhibit the bitterness of food and drugs (11, 12). PA was also used to stabilize liposomes encapsulating an emulsion nanodroplets used for ultrasonically activated drug delivery (13). A number of studies have revealed a decreasing toxicity of amphotericin B administered in the vesicles, liposomes, micelles and emulsions based on negative charged phospholipids, like PA and phosphatidylglycerol (14, 15). PA monosodium salt was also examined as an additive in cationic arginine glyceride conjugates which were used as cationic surfactants (16). PA can be easily prepared by phospholipids hydrolysis using plant extracts with phospholipase D (PLD). PLD was first isolated from carrot (17), is widespread in plants, animals and microorganisms and is well known for hydrolytic and transphosphatidylation activity towards natural phospholipids and their synthetic analogs. PLD catalyzes hydrolysis of the terminal phosphodiester bond on phospholipid giving PA and free aminoalcohol group. In the presence of an another alcoholic nucleophile, PLD catalyzes exchanging the polar head group, giving a new phosphoester bond (18-20). Many previous studies, using crude and purified, free or immobilized PLD from plant and microbial, showed that this enzyme is capable of hydrolyzing a broad range of phospholipids. However, significant differences of the substrate for PLD may occur, PC is assumed as the best preferable substrate for this enzyme. It is well known that PLD activity depends on source and purity of enzyme, structure and form of phospholipid, concentration of Ca^{2+} and pH level. The most important difference in phospholipid structure (from different sources) is their fatty acids profile, especially length and degree of unsaturation of carbon chain. It is known from previous studies on egg, soybean and synthetic phospholipids that the degree of fatty acids saturation in phospholipids have influence on the PLD activity. PLD from cabbage and Streptomyces sp. are exploited for a number of phospholipid-transforming reactions in the laboratory as well as on industrial scale (21-23). Phospholipids from soya and egg yolk are used as a source of natural PA. Although hydrolytic activity of PLD from different sources in hydrolysis of egg, soybean and synthetic phospholipids is well described in many studies, activity of cabbage PLD extracts in hydrolysis of rapeseed phospholipids has not been reported. Lower sensitivity to oxidation of rapeseed phospholipids compared to soya ones give a chance to use them as a more stable phospholipids derivatives. Furthermore, there is no standards for rapeseed PA, as yet. For that reasons, in our study the hydrolysis of rapeseed phosphatidylcholine (RPC) to rapeseed PA (RPA) in a biphasic system was examined. Purified by us PLD extracts, from three varieties of cabbage were used and the results were compared with standard cabbage phospholipase (PLD_{st}).

MATERIALS AND METHODS

Enzymes and chemicals

PLD_{st} from cabbage (type IV, activity 335 units/mg solid, liberated 1 μmol/h choline from egg at pH 6.5 and temp. 30°C) and egg PA sodium salt (purity 99%) were from Sigma-Aldrich (St. Louis, MO, USA). Solvents of HPLC grade and TLC silica gel G 60 plates were purchased from Merck (Darmstadt, Germany). All other solvents and reagents of analytical grade were from POCH (Gliwice, Poland).

Preparation of rapeseed phosphatidylcholine

Crude commercial rapeseed lecithin, free of erucic acid and glucosinolates (00-type rapeseed), was obtained from the Company Kruszwica SA (Brzeg, Poland). The raw material was deoiled with acetone and fractionated with 95% ethanol, to obtain rapeseed lecithin ethanol soluble fraction, by the method described elsewhere (24). Further purification of lecithin ethanol soluble fraction was performed by column chromatography on aluminum oxide as previously described (25). As a result the RPC with 99% purity (HPLC) was obtained.

Preparation of phospholipase D cabbage extracts

Fresh cabbage varieties: savoy cabbage (*Brassica oleracea* var. *sabauda*), white cabbage (*Brassica oleracea* var. *capitata f. alba*) and brussels sprouts (*Brassica oleracea* var. *gemmifera*), were obtained from the local market. Crude PLD extracts were prepared by the method of Davidson and Long (26). Hundred grams of cabbage leaves or sprouts were homogenized with 200 mL of water at 4°C for 5 min at 4000 rpm (MPV 120 Homogenizer, MPV MED Instruments, Poland). Homogenate was stored at 4°C for 60 min and filtered. After that, the homogenate was centrifuged twice at 4000 rpm for 15 min at 4°C (MPV 325 centrifuge MPV MED Instruments, Poland). The supernatant was heated at 50°C for 5 min, immediately cooled, and centrifuged as described. Finally PLD extracts were freeze-dried at -50°C, 0.03 mBar (Freezone VI, Labconco, USA) to yield a stable pale beige powder.

Cabbage extracts hydrolytic activity

Hydrolytic activities of cabbage PLD extracts towards RPC were compared to activity of standard PLD. Hundred milligrams of RPC was dissolved in 10 mL of diethyl ether and added to the mixture of 0.4 mg PLD_{st} or 400 mg cabbage PLD extract, 40 mM $CaCl_2$, in 10 mL of acetate buffer (pH 5.6), and then incubated at 30°C under stirring for 30 min. Product of hydrolysis was extracted three times with 40 mL of diethyl ether. Extracts were combined, dried with anhydrous sodium sulfate and evaporated to dryness at 40°C under vacuum. The phosphatidylcholine and PA content was analyzed by TLC and HPLC as a 1% w/v solutions in chloroform.

Hydrolysis of rapeseed phosphatidylcholine

Hundred milligrams of RPC was dissolved in 10 mL of diethyl ether and added to the mixture of 200-1000 mg PLD extract from savoy cabbage, 20-160 mM $CaCl_2$ in 10 mL of acetate buffer of pH 5.6. The obtained mixture was then incubated at 30°C under stirring for different times in the range of 15-120 min. Next, the product of hydrolysis was extracted three times by 40 mL of diethyl ether. Extracts were combined, dried with anhydrous sodium sulfate and evaporated to dryness at 40°C under vacuum. The PC and PA content was analyzed by TLC and HPLC as a 1% w/v solutions in chloroform.

TLC analysis

TLC was performed on 20 × 20 cm silica gel plates. Samples of 20 μL of chloroform phospholipids solutions were applied and the plates were developed with chloroform-methanol-water (45 : 25 : 0.2, v/v/v) in saturated glass chamber to 15 cm high. The plates were sprayed with 5% phosphoromolybdate ethanol solution and dried at 105°C.

HPLC analysis

Further calculation of the products was performed by HPLC analysis using a Waters 600 unit, fitted with a UV photodiode array detector Waters 996 PDA and a data processor (Millenium 32, Waters). The chromatograms were evaluated at 205 nm. A 250 × 5 mm column packed with Lichrospher 100-10 (Knauer) was used. Column flow rate was 1 mL/min and the column temperature was maintained at 20°C. Phospholipids were separated by isocratic elution with hexane/2-propanol/water at ratio of 1 : 4 : 1, v/v/v. Samples of 10 μL (1% w/v) were injected in the same solvent used for elution. The peaks were identified on the basis of their retention times using standard phospholipids. PA concentration was calculated as a peak area from the standard curve.

RESULTS AND DISSCUSION

We have characterized the hydrolytic activity of cabbage PLD extracts towards RPC. The partially purified enzyme extracts have been used, and the routine purification has not been continued beyond heat precipitation step. The purified by us cabbage PLD acts readily upon PC (22, 26, 27). Cabbage PLD of the highest purity proved rather unstable and the purification was accompanied by significant reduction of enzyme activity and yield (26-28).

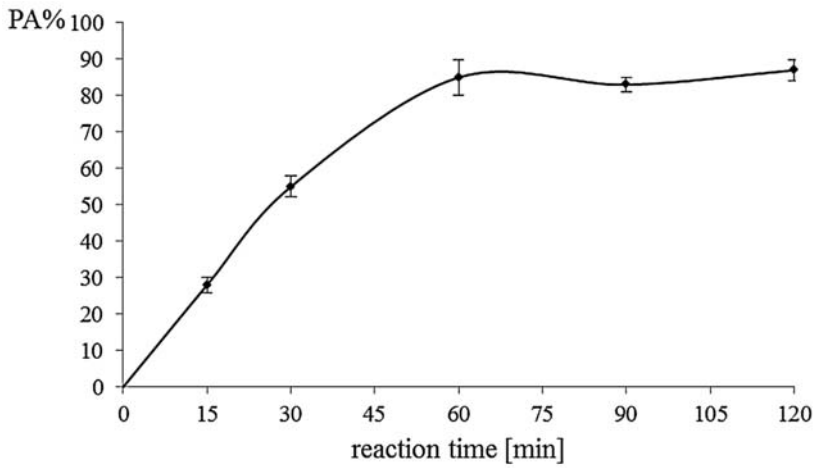

Figure 1. Effect of reaction time on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, PLD_{sc} 400 mg, 30°C). The calculations are given as the mean values of triplicate measurements

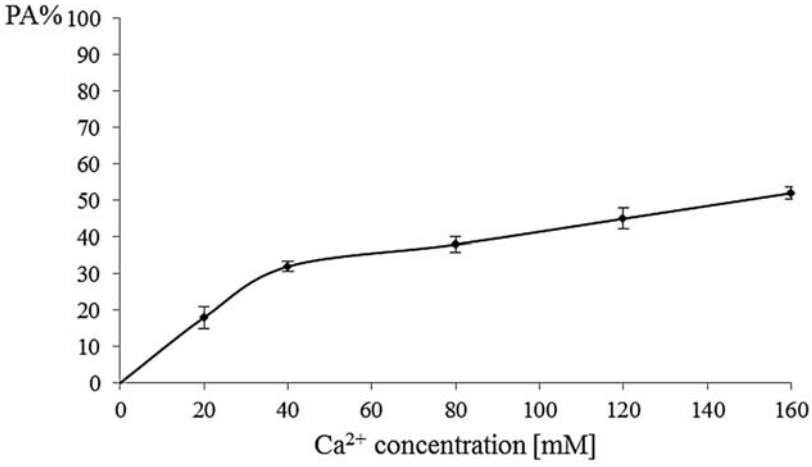

Figure 2. Effect of Ca^{2+} concentration (mM) in reaction mixture on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, PLD_{sc} 400 mg, 30°C, 60 min). The calculations are given as the mean values of triplicate measurements

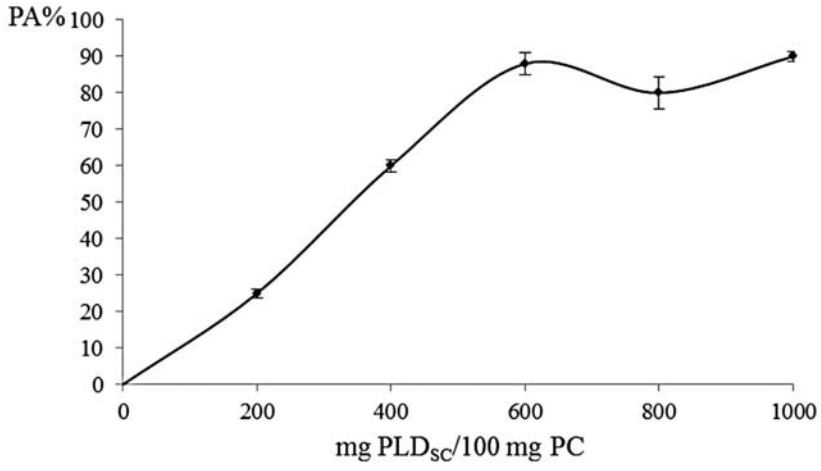

Figure 3. Effect of cabbage phospholipid D extract amount (mg PLD_{sc}/100 mg RPC) on phosphatidic acids concentration (PA%) in hydrolyzed RPC (RPC 100 mg, Ca^{2+} concentration 120 mM, 30°C, 60 min). The calculations are given as the mean values of triplicate measurements

Hydrolytic activity of crude cabbage phospholipase D

Biphasic system with the diethyl ether as a phospholipids solvent was used. Substrate and product differ in their R_f values, for RPC and RPA spots of 0.25 and 0.61, respectively. As shown in Table 1, all of cabbage extracts have PLD hydrolytic activity towards RPC. The highest calculated activity exhibit PLD extract from savoy cabbage (4.17×10^{-3} [mg PA/min × mg PLD extract]). This enzyme extract was used in all the following experiments. The results are in accordance with hydrolytic activity of cabbage PLD towards egg, soya and synthetic PC. Some reports have demonstrated hydrolytic activity of savoy cabbage extracts towards many natural and synthetic phospholipids, especially PC (21, 22, 26, 28) but not of RPC.

Effect of reaction time on rapeseed phoshatidylcholine conversion to rapeseed phosphatidic acid

It is known that the amount of PC hydrolyzed to PA is strongly dependent on physicochemical properties of reaction mixture. Generally, PC is regarded as the best, readily hydrolyzed substrate for cabbage PLD. The reaction time of the RPA formation in our study was examined in the range of 15-120 min. As shown in Figure 1, TPA level is rapidly increased up to 60 min of reaction time, and

Table 1. Hydrolytic activity of phospholipase D cabbage extracts from white cabbage (PLD_{wc}), savoy cabbage (PLD_{sc}), brussels sprouts (PLD_{bs}) compared to standard phospholipase D (PLD_{st}) towards rapeseed phosphatidylcholine (RPC 100 mg). (PA – phosphatidic acid, $CaCl_2$ concentration 40 mM, temperature 30°C, reaction time 30 min).

PLD extract		PA content in the hydrolysis product [mg]	PLD extract activity [mg PA/min × mg PLD extract]
Kind of cabbage	Amount [mg]		
PLD_{wc}	400	45	3.75×10^{-3}
PLD_{sc}	400	50	4.17×10^{-3}
PLD_{bs}	400	28	2.33×10^{-3}
PLD_{st}	0.4	90	7.50

The calculations are given as the mean values of triplicate measurement.

then is almost constant, probably because of PLD inhibition by formed PA. Fast hydrolysis of soya PC by cabbage PLD in biphasic system was also observed in previous studies (29). For the next experiments, 60 min time of hydrolysis was chosen.

Effect of calcium ions concentration on rapeseed phosphatidylcholine hydrolysis to rapeseed phosphatidic acid

PLD is a metal dependent enzyme. Especially Ca^{2+} concentration play a crucial role in plant enzyme activation (30). In present study, Ca^{2+} concentrations were determined in the range from 20 to 160 mM. As shown in Figure 2, the optimum activity of PLD extract from savoy cabbage towards RPC was observed at 120 mM Ca^{2+}. This result is somewhat higher than it was observed for another plant PLD used in other study (30), where used Ca^{2+} concentration was up to 100 mM. It may be due to fatty acid profile and to substrate (RPC) concentration in the reaction mixture. The effective Ca^{2+} concentration as a dependence on substrate amount was previously observed (26). Similar Ca^{2+} concentration (120 mM) was reported in the enzymatic hydrolysis of egg yolk PC by rapeseed phospholipase D extract (31).

Effect of phospholipase D/rapeseed phosphatidylcholine ratio on rapeseed phosphatidylcholine conversion to rapeseed phosphatidic acid

RPA concentration in hydrolyzed RPC in experiments with various enzyme/substrate ratios from 200 to 1000 mg PLD_{sc}/100 mg RPC are shown in Figure 3. From the previous study where cabbage and garlic phospholipase D extracts were used (23) it is known that substrate concentration does not directly affect the hydrolysis rate. But there was observed a strong interdependence of substrate form (monomeric or aggregated forms), their critical concentration in reaction mixture and activation by Ca^{2+} (23, 27). In our study, it is shown (Fig. 3) particularly significant conversion of RPC to RPA observed at enzyme/substrate ratio 600 mg PLD_{sc}/100 mg RPC. Enzyme/RPC ratios over 600 mg PLD_{sc}/100 mg RPC was not significant for RPA content in the hydrolysis product of RPC. Pantazi et al. have observed a significant decrease in the PA amount formed in the hydrolysis of synthetic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine applying in the study a constant amount of enzyme extract and increasing quantities of phospholipids substrate (22).

Lambrecht and Urlich-Hoffman (27) have observed, in the study of short chain egg PC hydrolysis with cabbage phospholipase D, occurrence of the inflection point in the effect of substrate amount on phospholipase D activity. Above this point, phospholipase D activity significantly increased. In our study, where was used RPC, such phenomenon was not observed. This may be caused by differences in the fatty acids profile of egg and RPC. RPC may form in the reaction mixture other forms of phospholipid aggregates, compared to egg phosphatidylcholine (32).

CONCLUSIONS

The hydrolytic activity of cabbage phospholipase D towards RPC has been reported. Our results show PLD_{sc} activity towards rapeseed phosphatidylcholine similar to phosphatidylcholine from egg, soya or synthetic. It is difficult to make a fair comparison, because a lack of information concerning the variety of cabbage used for extracts with phospholipase D preparation and the differences in fatty acids profiles of rapeseed phosphatidylcholine compared to the profiles of substrates phosphatidylcholine used in other studies.

In conclusion, presented study shows that the hydrolysis of RPC to RPA using purified by us PLD_{sc} gives a product, with the satisfactory conversion degree up to 90% and it is not necessary to use expensive, high purity phospholipase D cabbage extracts.

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TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES OF THE EXTRACT OF *EPHEDRA PROCERA* FISCH. ET MEY

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Abstract: *Ephedra procera* belonging to the family Ephedraceae is a poison and medicinal plant. The main aim of present study was to determine total phenolic content and antioxidant and antibacterial activities of ethanolic extract from the aerial parts of *E. procera* collected from a natural habitat in Chaharmahal va Bakhtiari province, Southwestern Iran. The total phenolic content of the extract by Folin-Ciocalteu method and the antioxidant activity using DPPH assay were determined. The antibacterial activity, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) of the extract were evaluated against five bacteria, including *Proteus vulgaris, Pseudomonas aeruginosa, Enterobacter aerogenes, Bacillus cereus* and *Staphylococcus aureus*. Total phenolic content in the extract of *E. procera* was 0.718 mg tannic acid/g dry weight extract. The results indicated that the ethanolic extract of *E. procera* exhibited radical scavenging activity. In addition, the results of this study confirmed that the ethanolic extract of *E. procera* exhibited antibacterial activity. In conclusion, the extract of *E. procera* could be an important source of phenolic components with antioxidant capacity and antibacterial activity.

Keywords: *Ephedra procera*, total phenolic content, biological activity

Ephedra L. (Ephedraceae) is a genus belonging to the Gnetales, the closest living relatives of the Angiosperms (1, 2). The family Ephedraceae has the only genus *Ephedra* L., which consists of about 50 species of perennials and shrubs in the world. *Ephedra* L. generally grows wild in arid and semiarid climates and is distributed mainly in the temperate zones of Europe, Asia and North America (3). The *Ephedra* consists of a group of perennial, evergreen, and dioecious sub-shrub species growing up to four feet tall, with slender and joined stems (4). The *Ephedra* species are called "joint-pine", "joint fir", "sea grape", "mormon-tea" or "shrubby horsetails" in English, and "Ormak", "Rish-boz" or "Ali-jonak" in Persian (5). In the flora of Iran, 12 species of *Ephedra* has been reported (5). This genus is commonly used by the Chinese people as a folk medicine for treatment of allergies, bronchial asthma, chills, colds, coughs, edema, fever, flu, headaches, and nasal congestion (6). Results of previous studies on biological activity of the plant indicted that the extract from aerial parts of the *Ephedra* species have antimicrobial and antioxidant activities (7-12) and anti-ulcer properties (3). The main chemical compounds have been identified and isolated from *Ephedra* extract as alkaloids group such as ephedrine, pseudoephedrine, and norpseudoephedrine (4, 13, 14).

Medicinal plants can contain a wide variety of free radical scavenging molecules, including phenolic compounds, terpenoids etc., which are rich in antioxidant activity (15-17). Phenolic compounds are an integral part of the human diet and could be helpful against cancers, arteriosclerosis, ischemia, and inflammatory disease, which are caused by exposure to oxidative stress (18).

To our knowledge, there are no published reports on total phenolic content, antibacterial and antioxidant activities of *Ephedra procerea* belonging to the family Ephedraceae. The main objective of this study was to evaluate the content of phenolic compounds, antioxidants and antibacterial activities of the extract from *E. procera*.

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EXPERIMENTAL

Plant material

The aerial parts of *E. procera* were collected from a natural habitat in Chaharmahal va Bakhtiari province, Southwestern Iran (latitude 31°N; longitude 50°E; altitude 2250 m above sea level) in April 2012 (Fig. 1). Identifications were consequently confirmed with the help of the authentic specimens deposited at the Herbarium, Research Center for Agriculture & Natural Resources, Chaharmahal va Bakhtiari province, Shahrekord, Iran (No. 3025). Soil physical and chemical characteristics of natural habitat, including pH, electrical conductivity (EC), organic carbon (OC%) and soil texture were determined (Table 1).

Chemicals and reagents

Tannic acid, Na_2CO_3, and ethanol used in this study were purchased from Merck Co. (Darmstadt, Germany). The Folin-Ciocalteu reagent, and the 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. (Steinheim, Germany).

Extract preparation

The aerial parts of the plant were shade dried and ground into a powder (100 g), macerated in 200 mL of ethanol 70% and filtered and then were dried at 35°C under rotary vacuum (Model Zirbus 302w, Italy). The extract samples were stored in universal bottles and refrigerated at 4°C prior to use.

Determination of total phenolic content

The total phenolic content in each extract was determined using the Folin-Ciocalteu method following procedure of Singleton and Rossi (19) with some modifications. Briefly, 0.5 mL of the sample were mixed with 2.5 mL of Folin-Ciocalteu's phenol reagent and kept for 5 min at 37°C. Then, 2 mL of saturated Na_2CO_3 (7.5%) was added and the mixture was brought to 10 mL with the addition of deionized-distilled water. The mixture was maintained at room temperature in the dark for 120 min and then the absorbance of the mixture was measured at 765 nm against a reagent blank using a UV–Vis spectrophotometer (Shimadzu Corp., Japan). Tannic acid equivalent (TAE) was used as the reference standard and the TPC was expressed as mg of TAE equivalents per gram of each extract on dry basis.

Antioxidant test

The DPPH radical scavenging activity of extract was determined using the method proposed by Hung et al. (20). The extracts at concentrations of 16 to 500 µg/mL were mixed with an equal volume of 0.2 mM ethanol solution of DPPH. The disap-

Table 1. Geographical and climate of natural habitats of *E. procera*.

Region	P	T	pH	E.C.	O. C.	Sand %	Silt %	Clay %
Rig mountain	13.4	37.5	7.58	1.291	1.872	18.5	42	39.5

E.C.: electrical conductivity (dS/m), O.C.: organic carbon (%), and Sand, Silt and Clay in %. Meteorological information was obtained from weather stations located within the study area and the surrounding zone; each value in the mean of 10 to 15 year data. Soil characteristics are based on average of samples taken from three farms in each region.

Figure 1. Aerial parts of *E. procera*

pearance of the DPPH after 30 min of incubation at room temperature was determined spectrophotometrically at 515 nm. Ethanol was used to zero the spectrophotometer. The absorbance of the DPPH radical without antioxidant served as the control and was measured daily. The amount of sample necessary to decrease the absorbance of DPPH by 50% (IC_{50}) was calculated graphically and the percentage inhibition was calculated according to the equation:

$$\% \text{ inhibition} = \left[\frac{A_{C(0)} - A_{A(t)}}{A_{C(0)}}\right] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t = 30 min. The food preservative butylhydroxyanisole (BHA) was used as positive control.

Antibacterial test

Five strains of bacteria, including *Proteus vulgaris, Pseudomonas aeruginosa, Enterobacter aerogenes, Bacillus cereus*, and *Staphylococcus aureus* were obtained from Food Microbiology Laboratory, Veterinary Medicine Faculty, (I.A.U.) Iran. The density of bacteria culture for the test was adjusted to 0.5 McFarland standards (1.0×10^7 c.f.u./mL) and measured using a spectrophotometer (Eppendorf, AG, Germany). The MIC values were evaluated using the broth serial dilution method according to standard methods (21). Bacterial strains were cultured overnight at 37°C in Mueller Hinton broth (MHB). The extract dissolved in 5% DMSO was first diluted to the highest concentration (250 µg/mL) to be tested, and then a series of twofold dilutions were made in a concentration range from 16 to 250 µg/mL in 10 mL sterile test tubes containing nutrient broth. After incubation at 37°C for 24 h, absorbance at 630 nm was used as a measurement of bacterial growth using a spectrophotometer (22).

The MBC of extracts were determined according to the MIC values, i.e., 5 µL from MIC tubes were transferred to agar plates and incubated at 37°C for 24 h. The MBC was referred to the minimum concentration of extracts with no viable bacteria. Experiments were performed in three different times.

RESULTS AND DISCUSSION

Total phenolic content

Total phenolic content was determined spectrometrically according to the Folin-Ciocalteu method and calculated as tannic acid equivalents (TAE). Total phenolics content for the extract of *E. procera* was 718 mg TAE/g DWE. Total phenol content (TPC) was determined in comparison with standard tannic acid. Rustaiyan et al. (23) reported that a total phenolic content of *Ephedra sarcocarpa* growing in Iran was 709.18 mg catechin equivalent/g extract. Moreover, in other study, Rustaiyan et al. (23) reported that total phenolic content of *E. laristanica* growing in Iran was 513.03 µmol gallic acid/g extract. Results of a study of Ghasemi Pirbalouti et al. (3) indicated that total phenolic content in the extract of *Ephedra pachyclada* collected from Kerman, Iran was 45 mg of GAE/g dry weight.

Antioxidant test

Antioxidant properties are very important in counteracting the deleterious role of free radicals in foods and biological systems. The potential antioxidant activity of the extract was determined by the scavenging activity of the stable free radical DPPH. This is a quick, reliable and reproducible method to assess the *in vitro* antioxidant activity of pure compounds as well as plant extracts (24). The DPPH is a stable free radical, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants (25-27). The effect of antioxidants on DPPH is based on their ability to donate a hydrogen atom to DPPH, thus converting the radical into a stable molecule (28). The lower IC_{50} value indicates a stronger ability of the extract to act as a DPPH scavenger while the higher IC_{50} value indicates a lower scavenging activity of the scavengers as more scavengers were required to achieve 50% scavenging reaction. In our study, the antioxidant activity of the ethanol extract from *E. procera* was expressed as IC_{50} with value 0.056 mg/mL, indicating that the extract acts as good DPPH scavenger. Rustaiyan et al. (29) reported that IC_{50} value in the DPPH assay of the methanolic extract of *Ephedra laristanica* growing in Iran was 4.6 mg/mL. Phenolics or polyphenols have received considerable attention, because of their physiological function, including antioxidant, antimutagenic, and antitumor activities (30). Plants phenolics present in herbs, because of their potential antioxidant activity, have been received considerable attention (31). Phenolic compounds, due to their antioxidant activities and free radical scavenging abilities, are widely distributed in plants (32), which have gained much attention and potentially have beneficial implications for human health (33). Therefore, phenolic compounds are the major group contributing to the antioxidant activity of vegetables, fruit, cereals and other plant-based materials. The antioxidant activity of phenolics is mainly due to their redox properties, which make them acting as reducing agents, hydrogen donors, and singlet oxygen quenchers (34).

Antibacterial test

The antibacterial activity of the ethanol extract of *E. procera* was tested against the five pathogenic bacteria, including *Proteus vulgaris, Pseudomonas aeruginosa, Enterobacter aerogenes, Bacillus cereus* and *Staphylococcus aureus* by using the serial dilution method. The extract demonstrated relative inhibitory activities against the pathogenic bacteria tested. The growth inhibiting activity varied according to the dose and bacterial strain. The MICs and MBCs of the tested samples are presented in Table 2. MBC values were mostly higher than MIC values. The results indicated that the different bacteria species demonstrated different levels of sensitivity to the extract. The MICs of the extract were within concentration ranges from 250 to 500 µg/mL, and the respective MBCs were 500 and > 500 µg/mL. Generally, the ethanol extract from *E. procera* indicated moderate to good inhibitory activities against five bacteria investigated. Antimicrobial activity of some *Ephedra* species, including *Ephedra altissima* Desf. (35), *Ephedra transitorai* (36), *Ephedra nebrodensis* (37), and *Ephedra breana* (38) has been noticed in recent years. Kwon et al. (39) reported the antimicrobial activity of *Ephedra sinica* extracts against bacteria, such as *Vibrio parahaemolyticus, Clostridium perfringens, Bacillus subtilis* and *Staphylococcus aureus*. In addition, Rustaiyan et al. (23) studied the antimicrobial capacity of the methanolic extract of *E. sarcocarpa* growing in Iran against Gram-positive and Gram-negative bacteria and fungi. Results of their study indicated that the extract of *E. sarcocarpa* inhibited the growth of Gram negative bacteria, being *Pseudomonas aeruginosa* (MIC = 16 µg/mL). Results of other study by Rustaiyan et al. (29) indicated the antimicrobial activity of the methanolic extract of *E. laristanica* growing in Iran. They reported that the extract of *E. laristanica* inhibited the growth of Gram negative bacteria, especially *Escherichia coli* (MIC = 32 µg/mL). Lee and Lee (10) reported that quinaldic acid isolated from the stems of *E. pachyclada* had antibacterial activity against *Clostridium difficile* and *C. perfringens,* while had no effect on the growth of *Bifidobacterium bifidum*, *Lactobacillus acidophilus* and *L. casei*.

Results from this study suggest that phenolic compounds are responsible of the antibacterial activity of extract of *E. procera*. Numerous works have reported the antibacterial effects of these metabolites against a wide range of bacteria (40-42). Phenolic compounds can act at two different levels: the cell membrane and cell wall of the microorganisms (43). They can interact with the membrane proteins of bacteria by means of hydrogen bonding through their hydroxyl groups, which can result in changes in membrane permeability and cause cell destruction. They can also penetrate into bacterial cells and coagulate cell content (44).

CONCLUSIONS

The present study is apparently the first report of quantitative total phenol profile, antioxidant and antibacterial activities of the ethanol extract from the aerial parts of *E. procera*. Results of this study indicated that the extract from *E. procera* had the highest antibacterial properties. Phenolic compounds present in the plant are responsible for its effective free radical scavenging, antioxidant and antibacterial activities. With regard to the results of this present study the extract of *E. procera* could be an important source of phenolic compounds with antioxidant capacity and antibacterial activity. Nonetheless, in order to gain better views on the antioxidant levels and activities in *Ephedra* species, further studies on purification, identification and quantification of each phenolic compound and other non-phenolic compounds are necessary in the future.

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Table 2. Antibacterial activity of extract of *E. procera*.

Pathogens	Gram	MIC (µg/mL)	MBC (µg/mL)
Proteus vulgaris	Negative	250	500
Pseudomonas aeruginosa	Negative	500	> 500
Enterobacter aerogenes	Negative	250	500
Bacillus cereus	Positive	250	500
Staphylococcus aureus	Positive	500	> 500

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PHARMACEUTICAL TECHNOLOGY

DISSOLUTION PROPERTIES AND KINETIC STUDY OF SULFADIMIDINE AND TRIMETHOPRIM TABLETS CONTAINING FOUR DIFFERENT SUPERDISINTEGRANTS

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Abstract: The objective of this study was to evaluate and compare the effect of four superdisintegrants such as croscarmellose sodium (Ac-Di-Sol), crospovidone (Kollidon CL and with smaller particle sizes Kollidon CL-F), sodium starch glycolate (Explotab) in combination with β-lactose and microcrystalline cellulose (Avicel PH-102) as base excipients exhibiting properties of directly compressed tablets and increasing the disintegration and the dissolution rate of sulfadimidine sodium (SDD-Na) and trimethoprim (TMP). All tablets were prepared by direct compression method and superdisintegrants were used at 2% for all formulations. The tablets were evaluated with regard to uniformity of weight, hardness, friability, drug content, disintegration time and dissolution properties. Dissolution properties such as $t_{50\%}$ and $t_{80\%}$ (time to release 50 and 80% of drug), $DP_{30,45}$ (percent of drug dissolved in 30 and 45 min) and the dissolution rate constant value (K) were considered in comparing the dissolution results. The results showed that crospovidone (Kollidon CL) provides the shortest disintegration time and the fastest rate of dissolution for both TMP and SDD-Na. The kinetic study of the dissolution data reveals that *in vitro* release profiles of TMP and SDD-Na can be best explained by first order model or by Higuchi model. The obtained data were plotted into Korsmeyer-Peppas equation to find out the confirmed diffusion mechanism. For TMP release, the values of the release exponent are beyond the limits of Korsmeyer model, so-called, power law. For SDD-Na release, exponent values are characteristic for anomalous transport (non-Fickian) or the value of the release exponent is beyond the limits of Korsmeyer model.

Keywords: dissolution, tablet excipients, sulfadimidine, trimethoprim, superdisintegrant, kinetic analysis

Solid dosage forms like tablets are the most popular and most preferred drug delivery systems. They have high patient compliance and they are relatively easy to produce and market in accurate dosing. Such a form presents good physical and chemical stability (1).

Despite the increasing interest in controlled-release drug delivery systems, the most common tablets are intended to be swallowed as a whole and to disintegrate and release their medicines rapidly in the gastrointestinal tract (2). Conventional tablet formulations generally require rapid disintegration to aid drug dissolution. The choice of formulation ingredients can have a significant effect on the rate and extent of drug dissolution (1). The simplest way to achieve quick disintegration is to use the superdisintegrant combined with suitable diluents.

The term superdisintegrant refers to a substance which achieves disintegration faster than the substances conventionally used. A tablet or a capsule content breaks up, or disintegrates, into smaller particles that dissolve more rapidly than in the case of the absence of such disintegrants (3). Superdisintegrants are generally used at a low level in a solid dosage form, typically from 2 to 5% of the weight of the total weight of a given dosage unit (4, 5).

A number of agents were formerly used as tablet disintegrants, but only a few acceptable disintegrants are currently available for pharmaceutical purposes (6). Superdisintegrants such as croscarmellose sodium, crospovidone and sodium starch glycolate are frequently used in tablet formulations to improve the rate and extent of tablet disintegration and thereby increase the rate of drug dissolution (7, 8).

Mechanism of disintegration

Despite all theories proposed, the mechanism of disintegration is still not completely understood.

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The rate of water uptake is of critical importance for a number of tablet disintegrants (9, 10).

However, no single mechanism is applicable to all disintegrating agents. It is likely that in most cases a combination of mechanisms take place simultaneously. The three major mechanisms affecting tablet disintegration include water uptake. The combination of swelling, wicking and deformation were found to be the primary action mechanisms for tablets disintegrants (5).

The most widely used veterinary antimicrobials in the European Union include tetracyclines, macrolides, penicillins, aminoglycosides and sulfonamides. In veterinary, sulfonamides are widely used to treat animals as well as to enhance feed efficiency, promote animal growth and improve productivity. They cover infectious diseases of the digestive and respiratory tracts, secondary infections, mastitis, metritis and foot rot (11–13). They are used in the treatment of otitis, bronchitis, sinusitis and pneumoystis pneumonia as well as in urinary tract infections in combination with trimethoprim (TMP) (14).

After the β-lactam class of compounds (among others containing penicillin), sulfonamides are the most commonly used antibiotics in most countries due to their ability to inhibit Gram-positive and Gram-negative bacteria as well as protozoa (15, 16). The objective of this study was to evaluate and compare the effect of four superdisintegrants such as croscarmellose sodium (Ac-Di-Sol), crospovidone (Kollidon CL and with smaller particle sizes Kollidon CL-F), sodium starch glycolate (Explotab) in combination with β-lactose and microcrystalline cellulose (Avicel PH-102) as base excipients exhibiting properties of directly compressed tablets and increasing the disintegration and the dissolution rate of sulfadimidine sodium (SDD-Na) and TMP.

EXPERIMENTAL

Materials

All chemicals were of analytical reagent grade. SDD-Na and TMP were purchased from P.O.Ch. S.A. (Gliwice, Poland). β-lactose (lactose) was purchased from Sigma, Germany. Microcrystalline cellulose (Avicel PH-102) and superdisintegrant – croscarmellose sodium (Ac-Di-Sol) were gift samples from IMCD (FMC Biopolymer, USA). Crospovidones (Kollidon CL and CL-F) were gift samples from BASF (Ludwigshafen, Germany) and sodium starch glycolate (Explotab) was a gift sample from JRS Pharma GmbH (Rosenberg, Germany). Magnesium stearate used as the internal lubricant was obtained from P.O.Ch. S.A. (Gliwice, Poland) and ethanol was from P.P.H.”STANLAB”. All the reagents and chemicals used were of AR analytical grade.

Water was purified by Cobrabid-Aqua CA-ROD 3 ECO system.

Methods

Blending and tableting

All tablets were prepared by direct compression method and the formulae used in the study are

Table 1. Formulation details of kinetic model for investigated tablets.

Formulation ingredients (mg/tablet)	Formula no.				
	F1	F2	F3	F4	F5
SDD-Na	89.9	89.9	89.9	89.9	89.9
TMP	16.7	16.7	16.7	16.7	16.7
Avicel PH-102	141.7	141.7	141.7	141.7	141.7
Lactose	141.7	141.7	141.7	141.7	141.7
Ac-Di-Sol	8	-	-	-	-
Explotab	-	8	-	-	-
Kollidon Cl-F	-	-	8	-	-
Kollidon Cl	-	-	-	8	-
Magnesium stearate (lubricant)	2	2	2	2	2
Total tablet weight (mg)	400	400	400	400	400

Table 2. Physical properties of SDD-Na and TMP formulations prepared.

Test	Results				
	F1	F2	F3	F4	F5
Mean weight (mg) (± %)	402 (1.5)	407 (2.5)	405 (2.7)	398 (1.8)	397 (1.3)
Thickness (mm) ± SD	5.0 ± 0.03	5.2 ± 0.02	5.2 ± 0.03	5.0 ± 0.04	4.9 ± 0.02
Hardness (kg/mm^2) ± SD	0.258 ± 0.04	0.266 ± 0.03	0.251 ± 0.03	0.255 ± 0.04	0.248 ± 0.05
Friability (%)	0.8	0.3	0.6	0.28	0.58
Disintegration time (min) H_2O ± SD	9.4 ± 0.75	10.5 ± 0.6	5.2 ± 1.2	4.2 ± 0.5	17.5 ± 1.7
Disintegration time (min) 0.1 M HCl ± SD	15.2 ± 1.7	17.5 ± 0.8	7.5 ± 1.1	4.4 ± 1.2	21.4 ± 1.4
Drug content (%) SDD-Na (%) TMP	100.74 99.32	98.44 97.62	100.85 98.75	98.44 98.15	99.52 101.35

shown in Table 1. Different types of super disintegrants such as Ac-Di-Sol, Kollidon CL and CL-F and Explotab were used.

Avicel PH-102 and β-lactose were used as diluents. SDD-Na and TMP were premixed with diluents and superdisitnegrant for 15 min in a cube mixer and then lubricated with magnesium stearate for another 5 min. The magnesium stearate level was fixed at 0.5% for all the formulations. Superdisintegrants were used at 2% for all the formulations.

The round flat-faced tablets were prepared using a single-punch tablet press (Erweka, EK-O, GmbH, Hausenstamm, Germany) with 9.0 mm punches.

Tablets properties

The tablets were evaluated as per standard procedure according to European Pharmacopoeia 7th edition (Ph. Eur.) for uniformity of weight, hardness, friability, drug content, disintegration time and dissolution properties (Table 2) (17).

Thickness and weight

Tablets were tested for thickness and weight variation to determine any variability associated with the tablet press or the method of preparation. Thickness was determined using digimatic caliper. Uniformity of mass was determined by weighing 20 tablets on an analytical balance (OHAUS Adventurer Pro).

Measurement of friability

Friability was evaluated from the percentage weight loss of 20 tablets tumbled in an Erweka TAR 120 friabilator (Erweka) at 25 rpm for 4 min. The tablets were dedusted and the loss in weight caused by fracture or abrasion was recorded as the percentage weight loss. Friability below 1% was considered acceptable.

Hardness test

The hardness of six tablets was determined using an Erweka TBH 30 hardness tester (Erweka). The hardness coefficient was calculated from equation:

$$T = \frac{P_{max}}{h \cdot d} \qquad \text{(Eq. 1)}$$

where: T – tablet hardness coefficient (kG/mm^2), P_{max} – tablet breaking force (kG), d – tablet diameter (mm), h – tablet thickness (mm).

All results are presented as the mean value ± SD (n = 6). A hardness coefficient above 0.1 kG/mm^2 was considered acceptable.

Disintegration time

Respective disintegration times of the prepared tablets were measured in 900 mL of purified water or 0.1 M HCl with disks at 37^{o}C using an ERWEKA ZT 222 tester.

The disintegration time (n = 6) was recorded till all the fragments of the disintegrated tablet passed through the screen of the basket.

In vitro dissolution test

The dissolution profiles of SDD-Na and TMP were determined in an Erweka DT 600 HH dissolution tester following the paddle method. All tests were conducted in 900 mL of purified water. The dissolution medium was maintained at a temperature of 37 ± 0.5^{o}C at a paddle rotation speed of 100 rpm. At specified time intervals (5, 10, 15, 30, 45 and 60

Table 3. Linear regression calibration formulae used for first derivative bivariate algorithm.

Component	Calibration equations and determination coefficients	
	λ = 249 nm	λ = 268 nm
SDD-Na	$^1D = –0.001761[SDD-Na] – 0.002453$ ($r^2 = 0.960$)	$^1D = – 0.161052[SDD-Na] – 0.010271$ ($r^2 = 0.999$)
TMP	$^1D = –0.164800[TMP] + 0.026986$ ($r^2 = 0.999$)	$^1D = 0.059386[TMP] + 0.021784$ ($r^2 = 0.999$)

Table 4. Recovery results for SDD-Na and TMP in the binary mixture applying the first derivative bivariate method.

TMP		SDD-Na	
Added (µg/mL)	Bivariate method (% found)	Added (µg/mL)	Bivariate method (% found)
2.0	102.7	2.0	99.9
4.0	100.1	4.0	98.8
8.0	96.7	8.0	100.1
12.0	99.5	12.0	100.6
16.0	98.1	16.0	99.6
20.0	97.2	20.0	98.9
% Mean recovery	99.1	% Mean recovery	99.6
%RSD (n = 6)	2.22	%RSD (n = 6)	0.71

Table 5. Wetting time and modified method disintegration time of prepared tablets.

Formula no.	Disintegration time H_2O (min) ± SD	Disintegration time HCl (min) ± SD	Wetting time H_2O (min) ± SD
F1	15.8 ± 0.6	18.4 ± 1.17	12.6 ± 3.5
F2	17.5 ± 0.9	21.3 ± 2.5	14.1 ± 2.2
F3	11.8 ± 1.5	12.7 ± 2.2	10.1 ± 2.7
F4	10.7 ± 1.1	11.1 ± 3.5	8.3 ± 1.7
F5	> 20	> 20	> 20

min), 2 mL of dissolution medium was withdrawn and replaced with an equal volume of purified water to maintain a constant total volume. The samples withdrawn were filtered through Whatman filter paper and SDD-Na and TMP content in each sample was analyzed after a suitable dilution by first derivative spectrophotometric method at λ = 249 nm and λ = 268 nm. Linear regression calibration formulae used for first derivative bivariate algorithm and recovery results for SDD-Na, and TMP in the binary mixture applying the first derivative bivariate method are shown in Tables 3 and 4 (18).

A Thermo Scientific Helios Omega UV-VIS spectrophotometer connected to PC fitted with VISION pro software was used for all the measurement and treatment of the data. The drug content in each sample was calculated using calibration equations. The dissolution rate was studied for the prepared formulations.

Drug content estimation

The powder content of 10 tablets from each formulation was mixed well and a powder sample equivalent to 89.9 mg of SDD-Na and 16.7 mg of TMP was placed in individual 100 mL volumetric flasks. Each drug was dissolved in 25 mL of ethanol. The resulting mixture was vortexed for 5 min and the volume was raised to 100 mL with ethanol. The solution was filtered and then the suitable dilution

was analyzed for the drug content by first derivative spectrophotometric method (18).

Wetting time and disintegration time

The wetting time of the tablets was measured using a simple procedure. Five circular tissue papers with a 10 cm diameter were placed in a Petri dish with a 10 cm diameter. Ten milliliters of water containing eosin, a water-soluble dye, was added to the Petri dish. A tablet was carefully placed on the surface of the tissue paper. The time required for water to reach the upper surface of the tablets was noted as the wetting time.

The disintegration time was measured using a modified disintegration method (n = 5). For this purpose, a Petri dish (10 cm diameter) was filled with 10 mL of water or 0.1 M HCl. The tablet was carefully put in the center of the Petri dish and the time of the tablet necessary to completely disintegrate into fine particles was noted (19). The wetting time and modified method disintegration time of the prepared tablets are shown in Table 5.

Drug release kinetics

To study the release kinetics of the drugs release profiles, data obtained from *in vitro* drug release studies were plotted in various kinetic models: zero order (Eq. 2) as the cumulative percentage of drug release *vs*. time, first order (Eq. 3) as the log of percent drug remaining to be released *vs*. time, and Higuchi model (Eq. 4) as cumulative percentage drug release *Vs* the square root of time.

The zero order rate describes the systems where the drug release is independent of its concentration.

$$Q = K_0 \times t \qquad \text{(Eq. 2)}$$

where Q is the amount of drug released in time t, K_0 is the zero order rate constant expressed in units of concentration (20).

The first order describes the release where the release rate is concentration dependent.

$$Log\ Q = Log\ Q_0 - Kt/\ 2.303 \qquad \text{(Eq. 3)}$$

where Q is the amount of the drug released in time t, Q_0 is the initial amount of the drug and K is the first order rate constant (21).

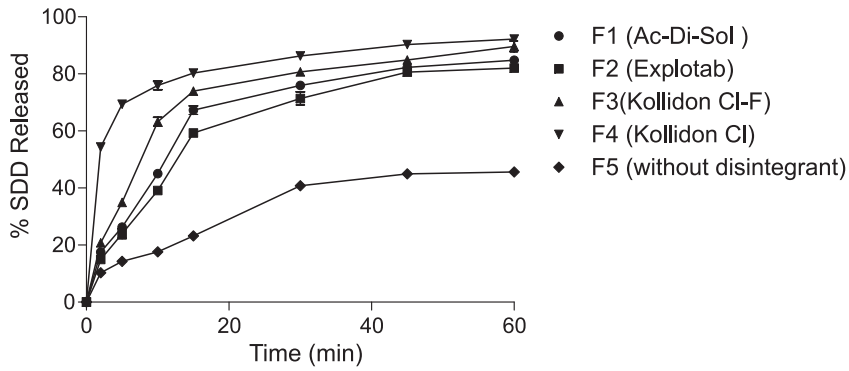

Figure 1. *In vitro* release profiles of sulfadimidine sodium from formulations: F1, F2, F3, F4, F5

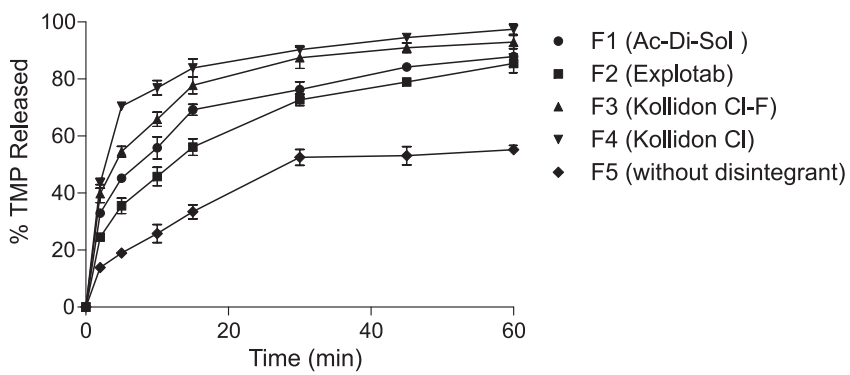

Figure 2. *In vitro* release profiles of trimethoprim from formulations: F1, F2, F3, F4, F5

Higuchi's model describes the release of drugs from insoluble matrices as a square root of time dependent process based on Fickian diffusion. The model, which is representative for the release of the soluble substances from the pharmaceutical formulas based on hydrophilic polymers, bases on the principle that the drug substance release profile decreases in time due to the increase in the length of the diffusion pathway followed by the drug substance.

$$Q = K\, t^{1/2} \qquad \text{(Eq. 4)}$$

where Q is the amount of the drug released in time t, K is the constant reflecting the design variables of the system (22).

Mechanism of drug release

To evaluate the mechanism of the drug release from tablets, the data of drug release were plotted in Korsmeyer-Peppas equation (Eq. 5) as the log of cumulative % of the drug released *vs.* log time, and the exponent n value was calculated through the slope of the straight line (23, 24) (Figs. 1, 2).

$$M_t / M_\infty = Kt^n \qquad \text{(Eq. 5)}$$

where Mt/M_∞ is the fraction of the drug released at time t, k is a constant incorporating the properties of the macromolecular polymeric system and the drug and n is an exponent used to characterize the transport mechanism. For cylindrical matrix tablets, if the exponent n = 0.45, then the drug release mechanism is Fickian diffusion, 0.45 < n < 0.89 for anomalous behavior or non-Fickian transport, n = 0.89 for Case II transport (relaxational), and n > 0.89 for Super Case II transport. Fickian diffusional release occurs by the usual molecular diffusion of the drug due to a chemical potential gradient. Case II relaxational release is the drug transport mechanism associated with stresses and state-transition in hydrophilic glassy polymers which swell in water or biological

Table 6. Drug transport mechanisms and diffusional exponents for cylindrical tablets.

Diffusional exponent, *n*	Type of transport	Time dependence
0.45	Fickian diffusion	$t^{1/2}$
0.45 < *n* < 0.89	Anomalous transport	t^{n-1}
0.89	Case II transport	time independent
n > 0.89	Super case II transport	t^{n-1}

Table 7. Time to release 50 and 80% of TMP ($t_{50\%}$ and $t_{80\%}$) and percent of drug dissolved at 30 and 45 min ($DP_{30,45}$).

Formula no.	$t_{50\%}$ (min) (mean ± SD)	$t_{80\%}$ (min) (mean ± SD)	DP_{30}(%) (mean ± SD)	DP_{45}(%) (mean ± SD)
F1	6.02 ± 0.4	38.38 ± 0.66	76.30 ± 2.78	84.2 ± 1.12
F2	13.09 ± 0.46	45.48 ± 0.28	72.67 ± 2.03	78.96 ± 1.26
F3	5.21 ± 0.22	25 ± 0.62	87.47 ± 3.80	90.93 ± 1.7
F4	4.11 ± 0.03	14 ± 0.33	90.32 ± 1.27	94.56 ± 0.15
F5	42.88 ± 0.77	-	52.51 ± 2.77	53.09 ± 3.23

Table 8. Time to release 50 and 80% of SDD-Na ($t_{50\%}$ and $t_{80\%}$) and percent of drug dissolved at 30 and 45 min ($DP_{30,}$ DP_{45}).

Formula no.	$t_{50\%}$ (min) (mean ± SD)	$t_{80\%}$ (min) (mean ± SD)	DP_{30}(%) (mean ± SD)	DP_{45}(%) (mean ± SD)
F1	15.11 ± 0.51	41.22 ± 0.67	75.93 ± 1.08	82.28 ± 2.11
F2	16.92 ± 0.61	44.89 ± 0.34	71.41 ± 4.00	80.61 ± 1.51
F3	11.09 ± 0.58	33.92 ± 0.88	80.70 ± 0.52	84.83 ± 1.32
F4	4.26 ± 0.09	22.21 ± 0.62	90.32 ± 1.27	94.56 ± 0.15
F5	-	-	40.77 ± 0.27	44.95 ± 2.41

fluids. Case II generally refers to the erosion of polymeric chain and anomalous transport (non-Fickian) refers to a combination of both the diffusion and erosion controlled drug release**.** To find out the mechanism of the drug release, the first 60% of the drug release data were fitted in Korsmeyer-Peppas model (23, 25).

Drug transport mechanisms and diffusional exponents for cylindrical tablets are presented in Table 6.

RESULTS AND DISCUSSION

Physical properties of tablets

The drug content of tablets was within the 100 ± 5% of the label claim and the results were satisfactory (Table 2). A good degree of uniformity of weight was achieved for all the batches of the tablet formulations prepared. The percent deviation did not exceed 5% indicating excellent uniformity of weight in all the batches of the tablet formulations prepared.

The tablet batches exhibited good mechanical properties with regard to both hardness and friability. The hardness values were above 0.1 kg/mm^2 and within the batches of tablet formulations they varied from 0.248 (kg/mm^2) for formulation F5 to 0.266 (kg/mm^2) for formulation F2. The fact that the strength of the tablets containing all the superdisintegrants was similar showed that the tablet hardness did not influence the dissolution. In the friability studies weight loss values of all the batches of tablet formulations were smaller than 1%.

The disintegration time was measured using a Ph. Eur. method and a modified disintegration method described above. The tablet formulations: F1, F2, F3 and F4 fulfilled the Ph. Eur. requirement for disintegration time for compressed tablets: less than 15 min. The order of disintegration times for the formulations of the tablets was: F4 < F3 < F1 < F2 < F5 (Tables 2, 5). The results of the Ph. Eur. disintegration time method correlated with both the modified disintegration time method and the wetting time. Significant prolongations of the disintegration time were observed for both sodium starch glycolate and croscarmellose sodium but not for crospovidone, a nonionic polymer. An acid medium significantly reduces the liquid uptake rate and capacity of ionic polymers.

In vitro dissolution studies

All tablet formulations were subjected to *in vitro* dissolution rate studies using purified water as the dissolution medium. Dissolution properties such as $t_{50\%}$ and $t_{80\%}$ (time to release 50 and 80% of drug), DP_{30}, DP_{45} (percent of drug dissolved at 30 and 45 min) and dissolution rate constant value (K) were considered in comparing the dissolution results. The corresponding values for SDD-Na and TMP tablet formulations are given in Tables 7, 8 and 9, 10. The dissolution profiles are shown in Figures 1 and 2. The results of the dissolution studies indicate that the dissolution rate of SDD-Na is increased in the following order: F5 < F2 < F1 < F3 < F4 and dissolution rate of TMP is increased in the same order: F5 < F2 < F1 < F3 < F4.

The dissolution rate of the model drugs correlated with the tablet disintegration time. Non-water-soluble crospovidones (Kollidon Cl and Kollidon Cl-F) provided the fastest dissolution in purified water for SDD-Na and TMP. Crospovidone is more effective than other superdisintegrants in enhancing the dissolution rate of poorly soluble TMP and water soluble SDD-Na. Crospovidone has solvent-like chemistry and high surface area resulting in high interfacial activity that enhances both the drug dissolution and release (4). The crospovidones act as disintegrants by absorbing water and subsequently swelling. This gain in volume is responsible for the disintegration of the tablet.

Water wicking and swelling are the two most important mechanisms of disintegrant action for croscarmellose sodium (Ac-Di-Sol). The cross-linked chemical structure of Ac-Di-Sol creates an insoluble, hydrophilic and highly absorbent excipient.

Derived from potato starch by cross linking, sodium starch glycolate (Explotab) demonstrates strong swelling properties in contact with water.

Despite their high hydration capacities, Ac-Di-Sol and Explotab were less effective in the tablet disintegration, probably their swelling formed a gel, which blocked tablet pores and prevented further penetration of water into the inner layers of the tablet (26). Crospovidone particles with their porous particle morphology quickly wick water into their capillaries to generate the rapid volume expansion and hydrostatic pressures that caused the tablet disintegration (27).

Kinetic analysis of dissolution data

The obtained drug release data were analyzed by zero order, first order, Higuchi and Korsmeyer-Peppas to know the mechanism of the drug release from the formulations. The release rate constants were calculated from the slope of the appropriate plot and determination coefficient (r^2) was determined (Tables 9 and 10).

Table 9. Dissolution kinetics of trimethoprim.

Formulation	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	K_0	r^2	K_1	r^2	K_H	r^2	n	r^2
F1	0.8488	0.8296	0.02833	0.9563	8.4462	0.9388	0.3213	0.9994
F2	0.9909	0.8934	0.02787	0.9823	9.6923	0.9859	0.4032	0.9978
F3	0.6874	0.6594	0.004271	0.962	7.0926	0.8024	0.2123	0.8729
F4	0.8011	0.76	0.3639	0.9305	8.132	0.8949	0.3147	0.996
F5	0.7915	0.8052	0.01082	0.8808	6.6244	0.9448	0.4223	0.9782

Table 10. Dissolution kinetics of sulfadimidine sodium.

Formulation	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	K_0	r^2	K_1	r^2	K_H	r^2	n	r^2
F1	1.0954	0.7687	0.00295	0.8982	11.712	0.9283	0.5799	0.9741
F2	1.1265	0.8227	0.02727	0.9275	11.52	0.9543	0.6694	0.9798
F3	0.5217	0.7427	0.0276	0.9267	5.2986	0.8757	0.1468	0.9533
F4	0.996	0.6874	0.03201	0.8847	11.728	0.8826	0.6838	0.9863
F5	0.7159	0.8609	0.00921	0.8951	6.1522	0.9468	0.471	0.9721

In this study, the *in vitro* release profiles of TMP from obtained tablet batches containing superdisintegrant were best explained by first order model as the plots showed the highest linearity and determination coefficient (r^2) was in the range from 0.9305 to 0.9823, followed by Higuchi model (r^2 equals from 0.8024 to 0.9859). The release of SDD-Na was best explained by Higuchi equation (r^2 equals from 0.8826 to 0.9543) followed by first order (r^2 equals from 0.8847 to 0.9275). This indicates that the release of the drug from matrix is a square root of time dependent process describing the drug release rate relationship with the concentration of the drug. The TMP and SDD-Na release profiles of the tablet batches without superdisintegrants showed best fit to Higuchi model (r^2 equals 0.9448 and 0.9468 for TMP and SDD-Na, respectively).

The obtained data were plotted according to Korsmeyer-Peppas equation to find out the diffusion mechanism.

For TMP release, the release profiles of tablet batches F1, F2, F4 and F5 showed good linearity (r^2) equals in the range from 0.9782 to 0.9994 and 0.8729 for F3 with exponent (n) values between 0.2123 and 0.4223. The value of the release exponent is beyond the limits of Korsmeyer-Peppas model, so-called, power law. Fickian diffusional release and a case-II relaxational release are the limits of this phenomenon.

For SDD-Na release, the release profiles of all the tablet batches showed good linearity (r^2) in the range from 0.9533 to 0.9833 with the release exponent (n) values in the range from 0.471 to 0.6838 and 0.1468 for tablet batch F3. This is characteristic for anomalous transport (non-Fickian), which appears to indicate a coupling of the diffusion and erosion mechanism or the value of the release exponent is beyond the limits of Korsmeyer-Peppas model.

CONCLUSION

The key is to choose superdisintegrant that would result in the maximum drug dissolution. The results of the present study conducted to evaluate the effect of crospovidone (Kollidon CL and CL-F), croscarmellose sodium (Ac-Di-Sol) and sodium starch glycolate (Explotab) on the dissolution rates of the model drugs: poorly soluble TMP and good soluble SDD-Na showed that Kollidon CL provides the shortest disintegration time and the fastest rate of dissolution for both TMP and SDD-Na.

The kinetic study of the dissolution data reveals that *in vitro* release profiles of TMP were

best explained by the first order model followed by Higuchi model. The release of SDD-Na was best explained by Higuchi's equation followed by first order. The data obtained were plotted into Korsmeyer-Peppas equation to find out the diffusion mechanism.

For TMP release, the values of the release exponent are beyond the limits of Korsmeyer model, so-called, power law. For SDD-Na release, the exponent values are characteristic for anomalous transport (non-Fickian) or the value of the release exponent is beyond the limits of Korsmeyer-Peppas model.

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EFFECT OF FORMULATION AND PROCESS VARIABLES ON THE RELEASE, MECHANICAL AND MUCOADHESIVE PROPERTIES OF IBUPROFEN TABLET FORMULATIONS

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Abstract: A 2^4 full factorial analysis was used to study the individual and interactive effects of binder type, X_1; binder concentration, X_2; relative density, X_3 and tabletting technique, X_4, on disintegration time (DT), brittle fracture index (BFI), tensile strength (TS) and mucoadhesion time (MT) of ibuprofen tablets formulated by direct compression (DC) and wet granulation (WG), and containing *Entandophragma angolense* gum (ENTA) as binder, in comparison with hydroxypropylcellulose. The result of the FTIR and UV peaks suggests the absence of any interaction between ENTA and ibuprofen. Interactions between the polymers and ibuprofen were determined using FTIR and UV determinations. The ranking of the individual effects on DT and BFI was $X_2 > X_3 > X_1 > X_4$, on TS; $X_3 > X_2 > X_1 > X_4$ and on MT; $X_2 > X_1 > X_4 > X_3$. The effects of changing the binder from hydroxypropylcellulose to ENTA led to an increase in DT and decrease in TS, BFI and MT. Changing X_2 and X_3 to higher values increased the DT and TS. The interaction between X_1 and X_2 had the highest influence on BFI and MT, while interaction between "X_3 and X_4", and "X_2 and X_3" had the highest influence on DT and TS, respectively. Ibuprofen tablets prepared by wet granulation method and containing *Entandophragma angolense* gum showed lower capping/lamination tendencies and better mucoadhesive drug release profiles.

Keywords: *Entandophragma angolense* gum, mechanical/mucoadhesive properties, ibuprofen, factorial analysis

The search for competitive alternatives to the expensive excipients incorporated during the formulation development of new drug entities or modification of already existing ones has led to the investigation of naturally sourced substances as excipients in drug delivery systems because they are less expensive, biocompatible and biodegradable (1, 2). Thus, naturally available polymers are being exploited for use as pharmaceutical excipients (3–5).

Mucoadhesive drug delivery systems are designed to prolong drug retention, thus offering advantages over conventional dosages through reduced dosage regimen and improved patient compliance. Mucoadhesion can be defined as a state in which two components are held together for extended periods of time by the help of interfacial forces, of which one of the components is of biological origin (6). The use of naturally occurring polymers such as *Entandophragma angolense* gum (Family: Meliaceae) as excipients in mucoadhesive drug delivery systems necessitates the evaluation of the mechanical and mucoadhesive properties of such formulations. Mechanical properties of tablets can be evaluated using tensile strength and brittle fracture index as parameters (7, 8), while mucoadhesive properties can be assessed (*in vitro*) using mucoadhesion time (9, 10). Factorial experimental design allows evaluation of the statistical significance of the main effects and the interactions between such natural polymers and selected drug candidates (11, 12).

Entandophragma angolense gum (Family: Meliaceae) obtained as exudates from the incised trunks of the tree, has been used extensively in battery electrolytes and printing inks as thickeners (13). Dark colored *Entandophragma angolense* trees produce a reasonable quantity of gum while the light colored trees lack the gum (14). The gum obtained by bark-slashing is somewhat sticky, faintly scented, and has a bitter taste (13). Adetunji et al. (15) reported the use of *Entandophragma angolense* gum as a suspending agent in oral formulations containing sulfamethoxazole.

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In the present work, a 2^4 full factorial design has been used to study the individual and interaction effects of nature of binder (denoted by N), concentration of binder (denoted by C), relative density (denoted by D) and tableting technique (denoted by M) on the disintegration time, tensile strength, brittle fracture index and mucoadhesion time of the tablets.

Ibuprofen has been chosen for this study because of its poor compressibility and hence it requires a binder among other excipients to form tablets of satisfactory tensile strength.

MATERIALS AND METHODS

The materials used were: ibuprofen powder BP, (BDH Chemicals Ltd., Poole, U.K.), lactose BP, magnesium stearate BP, and hydroxypropylcellulose (HPC) (Aqualon, Hercules Incorporated, USA), all supplied by Bond Pharmaceuticals Ltd., Nigeria. *Entandophragma angolense* gum (family: Meliaceae) was obtained from the incised trunk of the tree available within the complex of the Forestry Research Institute of Nigeria, Jericho, Ibadan, Nigeria.

Collection and purification of gum extract

The brown colored gum, collected as early morning exudates from previous incisions made on the trunk of *Entandophragma angolense* tree was weighed, allowed to dry and then thoroughly washed in chloroform/water (D/S) to remove associated earth particles. The precipitated gum was filtered, washed with diethyl ether and then dried in a hot air oven at a temperature of 40°C for 24 h. The dried gum was pulverized and passed through a number 60 mesh sieve (250 µm) (10, 16). The percentage weight of the purified and dried gum obtained from the exudates was then calculated. The dried gum (0.005 g) was dissolved in water, mounted on the microscope and observed for the presence of any foreign organic matter to determine the level of gum purity (17).

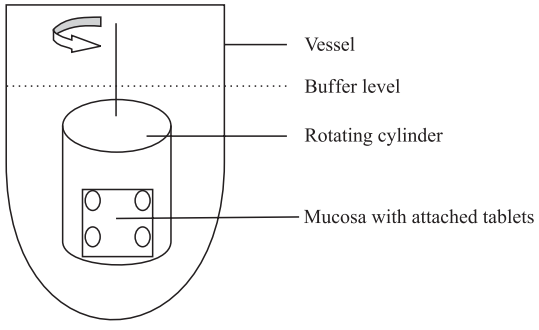

Figure 1. Rotating cylinder apparatus for mucoadhesion studies

Fourier Transform Infrared (FTIR) analysis

Spectra were obtained for the *Entandophragma angolense* gum, gelatin or HPC and ibuprofen using a Nicolet Magna-IR, 560 spectrometer.

A quantity (5 mg) of each of the completely dried powdered samples was weighed and then dispersed in 200 mg potassium bromide (pellet procedure). Signal averages were obtained at a resolution of 4 cm^{-1}.

UV analysis

Various mixtures of *Entandophragma angolense* gum or hydroxypropylcellulose and or ibuprofen were scanned in the wavelength range 190-300 nm. The maxima at 265 nm and 221 nm were monitored for wavelength shifts on a model DU-7400 spectrophotometer (Beckman, Fullerton, CA).

Preparation of granules

Batches (250 g) of a basic formulation comprising of ibuprofen, lactose, and *Entandophragma angolense* gum (or hydroxypropylcellulose), at a ratio of 6 : 3 : 1, respectively, were dry mixed for 5 min in a planetary mixer (Model A120, Hobart Manufacturing Co., U.K.) and moistened with appropriate amount of paste of the binding agent *Entandophragma angolense* gum (or hydroxypropylcellulose) to produce samples containing different concentrations of the binder.

Massing was continued for about 5 min and the wet masses were granulated by passing them manually through a no. 12 mesh sieve (1,400 µm). The granules were dried in hot air oven for 16 h at 60°C. The dried granules were then re-sieved through a number 16 mesh sieve (1,000 µm), before they were stored in air-tight containers.

Preparation of tablets

Wet granulation

Granule size fractions (500–1,000 µm) were used to prepare the tablets (400 ± 5 mg) using a Carver hydraulic hand press (model C, Carver Inc, Menomonee Falls, Wisconsin, USA), equipped with a 10.5 mm flat faced punch and die set lubricated with a 1% dispersion of magnesium stearate in acetone prior to compression.

Different compression pressures were employed to obtain different relative densities, ρ_r,

Table 1. Formulae for compressed tablets.

Formulations (% w/w)	A	B	C	D	E
Ibuprofen	5	5	5	5	5
Polymer (*Entandophragma angole*nse or hydroxypropylcellulose)	-	2.5	5.0	7.5	10.0
Talc	2	2	2	2	2
Spray dried lactose	93	90.5	88	85.5	83

for the tablets. The tablets were stored over silica gel for 24 h to allow for elastic recovery and hardening prior to measuring their weights and dimensions. The packing fractions (relative densities), ρ_r, of the tablets were calculated using the equation:

$$\rho_r = W / V\rho_s \quad (1)$$

where V = volume of tablets, W = weights of tablets, ρ_s = particle density of formulation.

Direct compression

The formulae for the compressed tablets are given in Table 1, and were prepared initially by premixing the *Entandophragma angolense* (or hydroxypropylcellulose) and ibuprofen for 15 min. Subsequently, lactose and talc were incorporated and the resulting composition was mixed for further 15 min. Compression was carried out at predetermined loads using a Carver hydraulic hand press, equipped with a 10.5 mm flat faced punch and die set lubricated with a 1% dispersion of magnesium stearate in acetone prior to compression.

Crushing strength and friability tests

Ten tablets from each formulation were tested for diametrical crushing test using the Erweka TBH 28 hardness tester (Apparatebau GmbH, Germany). Measurements were made in quadruplicate and the crushing strength results were accepted only if the samples split clearly into two halves. Tablet friability was determined using the Veego tablets friability apparatus (Veego Scientific Devices, Mumbai, India).

Determination of tensile strength and brittle fracture index

The tensile strength of the normal tablets (T) and apparent tensile strength of those containing a hole (T_o) were determined at room temperature by diametral compression (Erweka TBH 28 hardness tester). Measurements were made in triplicate on individual tablets and the results accepted only if the samples split clearly into two halves. Tensile strength (MNm^{-2}) calculated from equation 2 (18):

$$T \text{ (or } T_o) = 2F/\pi dt \quad (2)$$

where F is the load (MN) needed to cause fracture, d is the tablet diameter (m), and t is the tablet thickness (m).

The brittle fracture index (BFI) of the tablets was calculated from T and T_o by equation 3:

$$BFI = 0.5\,[(T / T_o)] - 1 \quad (3)$$

where T and To are as defined above (5, 18).

Disintegration test

Tablet disintegration time was determined in distilled water, at 37 ± 0.5°C with the Apex disintegration testing apparatus (Apex Construction Ltd.; Northfleet, Gravesend, Dartford, Kent, UK). Determinations were made in triplicate.

Mucoadhesion studies

Mucoadhesion studies were carried out to determine the time of detachment (mucoadhesive strength) of ibuprofen tablets attached to freshly excised intestinal mucosa of pig.

The rotating cylinder method, which is a slightly modified dissolution apparatus described in the United States Pharmacopoeia (USP) was used (Fig. 1). An intestinal segment of the mucosa was fixed on a stainless steel cylinder with the basolateral side facing the cylinder. The tablets were pressed on the apical side and the cylinder was transferred into a medium containing 500 mL of phosphate buffer, pH 7.4. The rotation speed was set to 60 rpm. The time taken for the tablets to detach from the mucosa was observed for tablets prepared by both wet granulation and direct compression techniques.

Factorial experimental design

To study the effects of nature of binder (denoted by N), concentration of binder (denoted by C) and relative density (denoted by D) and tableting technique (denoted by M) on the disintegration time, tensile strength, brittle fracture index and mucoadhesion time of the tablets, the experiments were performed based on the statistical modulation proposed by Woolfall (11). The basis of the experiment was to utilize a two-level factor using the four variables,

that is 2^4, and maintain a factorial structure. The levels are "high" level (denoted by the subscript H) and "low" level (denoted by the subscript L)

Using the above nomenclature, the expected combinations are represented by the following:

$N_H C_H D_H M_H$, $N_H C_L D_L M_L$, $N_H C_H D_L M_L$, $N_H C_H D_H M_L$
$N_H C_L D_H M_L$, $N_H C_L D_L M_H$, $N_L C_H D_H M_H$, $N_L C_L D_H M_H$
$N_L C_L D_L M_H$, $N_L C_L D_L M_L$, $N_L C_H D_L M_H$, $N_L C_H D_L M_L$
$N_H C_H D_L M_H$, $N_H C_L D_H M_H$, $N_L C_L D_H M_L$, $N_L C_H D_H M_L$

Where: N_L = Nature of polymer (*Entandophragma angolense*)

N_H = Nature of polymer (Hydroxypropylcellulose)

C_L = Concentration of polymer (2.5% w/w)

C_H = Concentration of polymer (10.0% w/w)

D_L = Relative density of tablet at 0.85

D_H = Relative density of tablet at 0.90

M_L = Direct compression technique

M_H = Wet granulation technique

By grouping the results into a number of sets, it was possible to assess the effect that each of the four variables had separately on the tensile strength and mucoadhesion time of the tablets. The effect of increasing the excipient N, from its 'low' level to its 'high' level on the disintegration time, tensile strength, brittle fracture index or mucoadhesion time can be determined by summing up all the values of tensile strength or mucoadhesion time of samples containing 'high' level of N and subtracting the sum of the values containing 'low' levels of N (11, 12).

Similarly, the effect of increasing the concentration of polymer (C), changing the relative density (D) or the compression technique (M) from 'low' to 'high' levels can also be determined using the adaptation of the method proposed by Woolfall (11).

To determine whether there was any interaction between two variables, the results of the combination in which they appear together at either high or low levels were summed and the sum of the other combinations were subtracted from this to obtain the interaction effects.

Montgomery (12) applied computer-based software to determine the various effects of these variables. The software (Minitab© 16), though based on the principles of Woolfall's work on product formulation (11), has been able to optimize performance in experimental processes and thus, reduced the manual calculation associated with previous processes.

Factorial experimental design allows estimation and testing of the statistical significance of the main effects and the interactions between factors. If two factors interact, then it implies that the effects of

Table 2. Values of disintegration time (DT), tensile strength (TS), brittle fracture index (BFI) and mucoadhesion time (MT) for the factorial experimental design obtained from the process parameters at low (L) and high (H) levels.

Combination codes				DT	TS	BFI	MT
N	C	D	M	(min)	(MNm^{-2})		(min)
L	H	H	H	7.71	1.291	0.277	205
H	H	L	L	7.42	0.992	0.223	308
L	H	H	L	7.39	1.799	0.268	241
L	H	L	H	6.22	1.271	0.219	209
L	L	H	L	2.09	1.779	0.158	176
H	L	H	H	2.47	1.812	0.182	207
H	H	H	H	8.37	1.331	0.290	322
H	L	H	L	2.76	2.113	0.173	200
H	H	H	L	9.71	1.798	0.281	313
L	L	H	H	2.92	2.156	0.169	214
L	H	L	L	5.96	1.123	0.217	233
H	H	L	H	6.83	1.198	0.232	321
H	L	L	L	1.94	0.817	0.122	181
L	L	L	H	1.63	1.173	0.118	203
H	L	L	H	2.10	0.982	0.131	204
L	L	L	L	1.45	1.017	0.109	184

N = nature of binder, C = concentration of binder, D = relative density of tablet, M = tabletting technique.

one factor depend on the setting of the other. Factor settings are very important in the presence of interactions since effects will not be additive in nature (12, 19, 20).

RESULTS

The results of determination of tensile strength, brittle fracture index, disintegration time and mucoadhesion time are given in Table 2.

Plots of formulation techniques and different concentrations of *Entandophragma angolense* gum on time of detachment of ibuprofen tablets are given in Figure 2. Formulations by wet compression generally show longer adhesion time than those by direct compression.

Plot of disintegration time for ibuprofen tablets (relative density of 0.9) containing different polymer concentrations and formulated using wet granulation (WG) and direct compression (DC) techniques are given in Figure 3.

The results of the factorial experimental design are given in Tables 3 and 4. A contour plot of tensile strength *versus* concentration of binder and nature of binder for tablets at a relative density of 0.90 formulated by direct compression is given in Figure 4.

DISCUSSION

The FTIR analysis showed the functional group region (4000 to 1300 cm^{-1}) having sharp peaks at 2926.85 cm^{-1} and 2853.19 cm^{-1}. These sharp peaks are characteristic of methyl C-H stretching associated with aromatic rings and carboxylic acids. The sharp peaks at 2359.93 and 2341.37 cm^{-1} are indications of asymmetric C-O stretch. The peaks obtained at 1573.69 and 1558.36 showed similar functional groups consisting of strong N=O nitroso and weak C-O stretch.

The fingerprint region consists of a characteristic peak at 1070.72 cm^{-1}. This peak confirms the presence of strong aromatic characters consisting of

Table 3. Summary of the individual coefficients of the variables on disintegration time (DT), tensile strength (TS), brittle fracture index (BFI) and mucoadhesion time (MT).

Variable factor	Coefficient	DT (min)	TS (MNm^{-2})	BFI	MT (min)
Nature of binder (X_1)	Effect p-value	0.389 0.018	-0.035 0.599	0.006 0.000	24.437 0.009
Concentration of binder (X_2)	Effect p-value	2.641 0.000	-0.065 0.338	-0.053 0.000	36.437 0.001
Relative density (X_3)	Effect p-value	0.617 0.001	0.344 0.000	0.027 0.000	2.188 0.780
Tabletting technique (X_4)	Effect p-value	-0.029 0.839	-0.014 0.834	0.004 0.000	3.063 0.697

Table 4. Summary of the interaction coefficients of the variables on disintegration time (DT), tensile strength (TS), brittle fracture index (BFI) and mucoadhesion time (MT).

Variable factor	Coefficient	DT (min)	TS (MNm^{-2})	BFI	MT (min)
X_1X_2	Effect p-value	0.242 0.035	0.015 0.762	22.563 0.001	43.13 0.001
X_1X_3	Effect p-value	0.011 0.905	0.040 0.437	1.312 0.718	0.15 0.718
X_1X_4	Effect p-value	-0.229 0.043	-0.036 0.475	3.437 0.363	1.00 0.363
X_2X_3	Effect p-value	0.227 0.043	-0.140 0.029	-0.938 0.796	0.07 0.796
X_2X_4	Effect p-value	-0.139 0.160	-0.064 0.227	-7.813 0.072	5.17 0.072
X_3X_4	Effect p-value	-0.306 0.732	-0.0984 0.086	-0.812 0.822	0.06 0.822

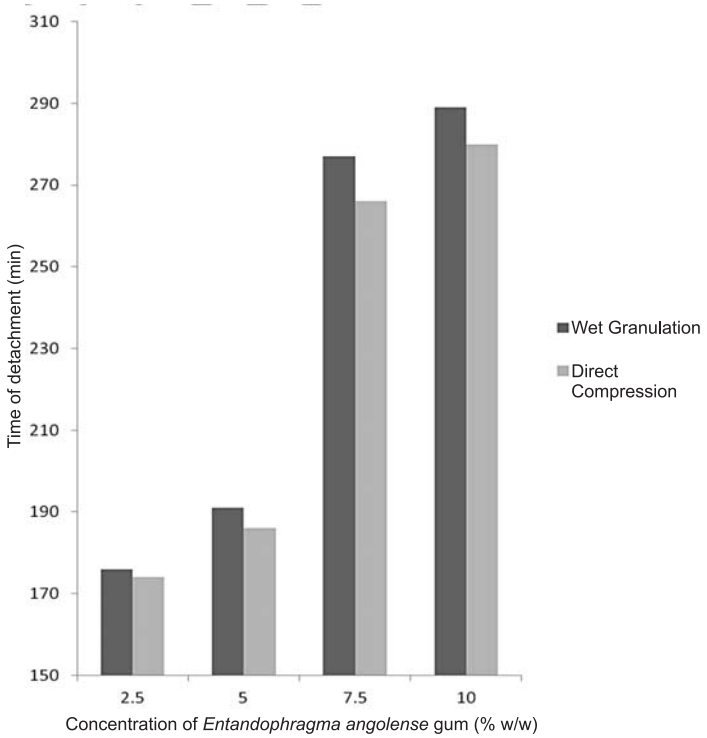

Figure 2. Plots of time of detachment (min) of ibuprofen tablets containing different concentrations of *Entandophragma angolense* gum formulated by wet granulation and direct compression techniques

C-O, C=O, C-N and C-F stretches, and weak P-H bending groups, which are present in materials like carbohydrates, starch, and natural polymers (21). The results revealed the presence of methyl, amine, phosphine and hydroxyl groups, in *Entandophragma angolense* gum. The presence of the characteristic peaks for the drug and polymers after mixing suggests the absence of a chemical reaction between *Entandophragma angolense* gum and ibuprofen.

The UV analysis of the various mixtures of *Entandophragma angolense* gum or hydroxypropylcellulose and/or ibuprofen showed no significant shift in wavelength of maximum absorption due to interaction between the polymers and the drug. The results of the peaks at wavelength range 190-300 nm for *Entandophragma angolense* gum powder in combination with ibuprofen powder at 221 nm suggest the absence of any reaction between *Entandophragma angolense* gum powder and ibuprofen.

The results of the factorial experimental design (Tables 3 and 4) provide a clear indication of the effects of the four independent process parameters: nature of binder (X_1), concentration of binder (X_2), relative density of tablet (X_3), and tabletting technique (X_4), on the four responses studied. The ranking of the individual effects on disintegration time was $X_2 > X_3 > X_1 > X_4$, on tensile strength, was $X_3 > X_2 > X_1 > X_4$ on BFI, $X_2 > X_3 > X_1 > X_4$ and on mucoadhesion time, $X_2 > X_1 > X_4 > X_3$. The rankings show the relative magnitudes of the effects of the factors on these variables. A positive effect signifies that the response variable has increased in value or magnitude, while a negative effect shows a decrease. Concentration of binder (X_2) had the largest positive effect on disintegration time of the tablets. This effect shows that changing the concentration from lower (2.5% w/w) to higher value (10% w/w) caused an increase in the disintegration time of the tablet formulations. This effect was significant ($p < 0.05$) and show that more compact tablets were formed as the binder concentration was increased, thus causing a reduction in the rate of disintegration. Two other factors (relative density of tablet, X_3 and nature of binder, X_1) also had positive effects on disintegration time. However, changing the binder from *Entandophragma angolense* gum to hydroxypropylcellulose caused an insignificant increase in both tensile strength and mucoadhesion time. Relative densi-

ty (X_3) had the largest positive effect on tensile strength of the tablets and the least effect on mucoadhesion time. This effect shows that changing the relative density from lower (0.85) to higher value (0.9) caused an increase in the tensile strength of the tablet formulations. This effect was significant ($p < 0.05$) and shows that more compact tablets were formed as the relative density was increased.

Brittle fracture index (BFI) is a measure of the tendency of a tablet to cap or laminate during decompression. It is measured by comparing the tensile strength (T_o) of a tablet with a central hole with the tensile strength (T) of a normal tablet. The hole is a built-in model defect that simulates the actual voids formed in the tablets (due to air entrapment or packing irregularities) during manufacturing (5). The voids or low density regions in a tablet are usually the weak points from which cracks emanate when stress (at the die wall) is applied to the tablet. The influence of X_2 (concentration of binder) on BFI was negative and the strongest. Hence, changing the concentration of the gum from lower (2.5% w/w) to higher value (10% w/w) causes a reduction in the BFI. This result shows that under the compressive forces employed in compaction, more of the polymer will facilitate more plastic deformation, to give tablets with reduced capping or lamination tendency.

Concentration of binder (X_2) had the highest positive effect on mucoadhesion. Mucoadhesion is a complex phenomenon which involves wetting, adsorption and interpenetration of polymer chains (22). The most critical stage in mucoadhesion is the development of strong adhesive bonds, which readily occurs as more binding sites are made available for attachment to the mucosa to occur due to increased polymer concentration (10). The implication of this is that increasing the concentration of the polymers had a direct relationship with the mucoadhesion of the tablets (Fig. 2). The polymers function as both binder and mucoadhesive, thus it is important to choose the optimum polymer concentration that will release the drug from the tablet matrix during adhesion.

Tensile strength was mostly affected by relative density of formulation (X_3) and the coefficient was positive, thus indicating that increasing the relative density led to an increase in the tensile strength of the formulations. This can be attributed to the fact that as the relative density of the tablet increases, more solid bonds are formed between the particles. This leads to increase in bond strength and hence, a subsequent

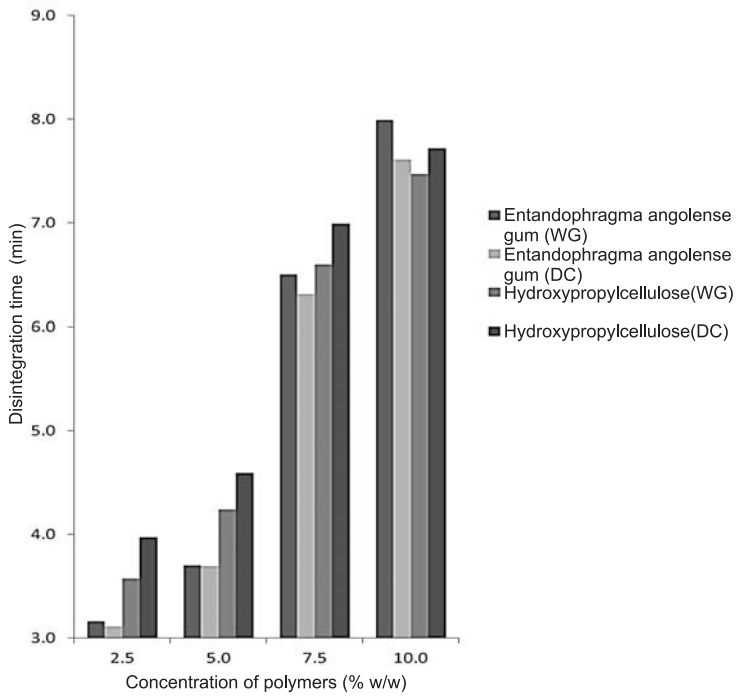

Figure 3. Plot of disintegration time for ibuprofen tablets (relative density of 0.9) containing different polymer concentrations and formulated using wet granulation (WG) and direct compression (DC) techniques

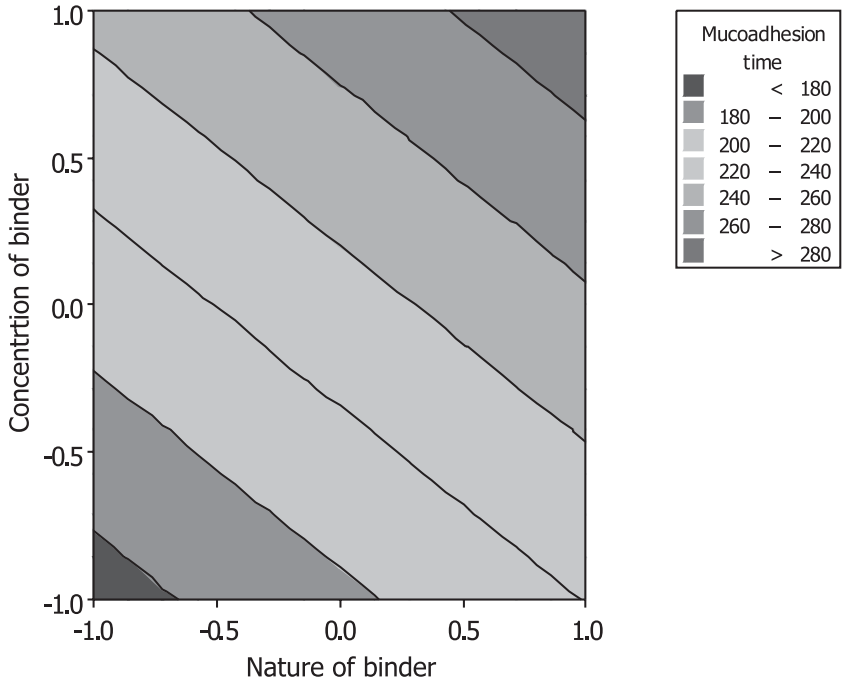

Figure 4. Contour plot of effect of interaction of concentration of binder and nature of binder on mucoadhesion time for tablets formulated by direct compression at a relative density of 0.90

increase in the tensile strength of the tablets (24). Tableting technique had a positive effect on mucoadhesion. The ability of polymers to swell in the presence of water could be responsible for the difference in the mucoadhesion time as a result of the different tableting techniques applied during the study. Thus, tablets formulated by wet granulation would adhere longer to the mucosa surface than those formulated by direct compression. This is as a result of the increased ionic strength of tablets formulated by wet granulation due to the presence of water, which will consequently enhance mucoadhesion (25).

The interaction coefficient values shown in Table 4 indicate the effects of the variable factors in combination. The ranking for the interaction effects on disintegration time was $X_3X_4 > X_1X_2 > X_1X_4 > X_2X_3 > X_2X_4 > X_1X_3$, on tensile strength, $X_2X_3 > X_3X_4 > X_2X_4 > X_1X_3 > X_1X_4 > X_1X_2$, on BFI, $X_1X_2 > X_2X_4 > X_1X_4 > X_1X_3 > X_2X_3 > X_3X_4$ and on mucoadhesion, $X_1X_2 > X_2X_4 > X_1X_4 > X_1X_3 > X_2X_3 > X_3X_4$. The results show that the interaction between the nature and concentration of the binder had the highest influence on mucoadhesion and BFI (Fig. 4), while the interaction between relative density and tabletting technique and between concentration of binder and relative density had the highest influence on disintegration time and tensile strength, respectively. Disintegration of tablets determines, to a large extent, the area of contact between the solid and liquid in the dissolution process. Many correlations have been made between disintegration time and parameters such as water penetration rate (26) and dissolution rate of tablets (27, 28). The disintegration time was mostly influenced by the interaction between tabletting technique and relative density of tablets. It was observed from the study that tablets formulated by the wet granulation technique had higher relative density and disintegration time values when compared with tablets formulated by direct compression (Fig. 3). The ability of polymers to swell in the presence of water could be responsible for the difference in the disintegration time as a result of the different tableting techniques applied during the study. The increased concentration of polymer binder which had the highest influence, in combination with relative density, on tensile strength can be attributed to the presence of more polymer particles available for bond formation and subsequently enhanced mechanical strength as characterized by the tensile strength values.

CONCLUSION

The FTIR and UV analyses suggest the absence of any interaction between *Entando-*

phragma angolense gum and ibuprofen. The variables employed in the formulations significantly affect the mechanical and mucoadhesive properties of the tablets formed. The rankings show that the greatest factor-factor interactions generally occurred between nature and concentration of the binder. This is probably due to the fact that the nature of the binder determines its plastoelastic properties and the degree of plastic deformation will undergo under compression forces. Ibuprofen tablets prepared by wet granulation method and incorporating *Entandophragma angolense* gum as binder showed lower capping/lamination tendencies and better mucoadhesive drug release profiles.

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PHARMACOLOGY

IN VITRO ANTIMICROBIAL ACTIVITY OF BRONCHOSOL

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Abstract: Bronchosol is a traditional medicinal product in the form of syrup used in cough and impeded expectoration. The active ingredients that it contains include extracts from the herb of thyme, the root of primrose and thymol. It is recommended in disorders of the respiratory tract when expectoration is impeded and secretion of liquid mucus in bronchi is insufficient. Antimicrobial activity of the components of Bronchosol, especially thyme and thymol, has frequently been reported in the literature. To date, there have not been any studies to confirm such activity of Bronchosol, though. The results of our research are the first one to point to the great activity of Bronchosol against microorganisms causing infections of the respiratory tract. It has been demonstrated that this product displayed antimicrobial activity against reference strains as well as strains of anaerobic and aerobic bacteria and fungi isolated from patients. The confirmation of the antimicrobial activity of Bronchosol provides an explanation of its effectiveness in the therapy of the respiratory tract infections.

Keywords: Bronchosol, antibacterial activity, antifungal activity, *Thymi herba, Primulae radix*

A cough is one of the most common symptoms of respiratory infections mostly of viral or/and bacterial etiology (1, 2). The main infectious factors include influenza and parainfluenza viruses, RS-virus, rhinoviruses and adenoviruses (3, 4). *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis* and *Streptococcus pyogenes* are primarily responsible for bacterial respiratory infections. They are also, yet less frequently, caused by Gram-negative baccilli, i.e., *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter spp.* and Gram-positive cocci from the *Staphylococcus aureus* species. Various species from the genera *Peptostreptococcus, Micromonas, Finegoldia, Prevotella, Porphyromonas, Fusobacterium, Bacteroides, Propionibacterium, Bifidobacterium, Leptotrichia, Tissierella* and *Selenomonas* are often enumerated among anaerobic bacteria that cause infections of the upper and lower respiratory tract. Sometimes, baccilli from the genus *Legionella* or atypical bacteria, i.e., *Chlamydia pneumoniae* and *Mycoplasma pneumoniae,* are the source of infection. On the other hand, yeast-like fungi, particularly from the genera *Candida albicans*, *Pneumocistis jiroveci* or protozoa, participate in such infections much more seldom (5-9). Great effectiveness and very high tolerance of preparations containing extracts from the herb of thyme and the root of primula have been shown in the treatment of respiratory infections, including acute bronchitis (10-12).

One such preparation is Bronchosol, syrup available on the Polish market since 2002. The active ingredients that it contains are a dense composite extract made of the herb of thyme, primula root and furthermore thymol. The herb of thyme (*Thymi* herba) and the root of primula (*Primulae* radix) are used in infections of the upper respiratory tract as agents that make expectoration of lingering mucus easier and stimulate secretion of liquid mucus in bronchi. The healing activity is connected with the presence of active compounds, mainly saponins, in the primula root and an essential oil found in thyme (8, 9, 13-17). *In vitro* studies have demonstrated that, apart from the activity making expectoration of lingering phlegm easier and anti-

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inflammatory activity (18, 19), the saponins from primula root show immunostimulant activity (8, 13, 15, 19, 20). Moreover, antimicrobial activity of extracts from primula root has been proven (19), but reports on this topic are sporadic. The essential oil as well as extracts from thyme show expectorant activity. It has been demonstrated that extracts from thyme and thyme oil decrease tension of bronchial smooth muscles and have anti-inflammatory activity (19, 21-24). A number of studies have concerned the antimicrobial activity of the extracts and essential oil from thyme. This activity is connected to a large extent with the presence of thymol, the main component of the essential oil (16, 20, 25, 26). It has been proven that perforation of bacterial cell membrane may be the key way of the antimicrobial activity of thymol. The measurement of the cell content outflow resulting from the activity of thymol has been made for bacteria found in the oral cavity, such as *Porphyromonas gingivalis, Selenomonas artemidis, Streptococcus sorbinus*. The decrease in intracellular ATP observed in *S. sobrinus* and *P. gingivalis* as well as the inhibition of ATP generation in *P. gingivalis* have been associated with the leak of the cell content (27). Great antimicrobial activity of the essential oil from the herb of thyme against bacteria causing respiratory infections most frequently, i.e., *Streptococcus pyogenes* (62 strains), *Streptococcus agalactiae* (20 strains), *Streptococcus pneumoniae* (6 strains) *Klebsiella pneumoniae* (6 strains), *Haemophilus influenzae* (25 strains), *Staphylococcus aureus* (5 strains), S*tenotrophomonas maltophilia* (5 strains), obtained from patients suffering from respiratory infections, has been demonstrated. *Haemophilus influenzae* and *Stenotrophomonas maltophilia* have been most susceptible to the activity of the oil from *Thymus vulgaris*, followed by *Streptococcus pneumoniae*. The *Klebsiella pneumoniae* strain has been least sensitive. The MIC and MBC values have been comparable and ranged from 0.025 mL/mL (*K. pneumoniae)* to 0.003125 mL/mL (*S. maltophilia*). The essential oil has shown antimicrobial activity against the *Streptococcus* spp. and *Haemophilus influenza* strains, which are resistant to the activity of numerous antibiotics, yet it has not been effective against the following antibiotic-resistant strains: *S. aureus*, *K. pneumoniae*, *S. maltophilia* (28). However, the results of the study by Tohidpour et al. (29) suggested antimicrobial activity of the *Thymus vulgaris* oil against methicillin-resistant strains of clinical bacteria isolated from wounds, eyeball and trachea, including *Staphylococcus aureus* (MRSA) and the REFERENCE strains of *Bacillus cereus*, *Escherichia coli*, *K. pneumoniae*, *S. aureus* and *S. aureus* MRSA. The essential oil from thyme inhibited the development of the reference strains. The MIC ranged from 0.1-4 v/v % (35). The essential oil and extracts from thyme have also shown antifungal activity (16, 20, 30).

MATERIALS AND METHODS

Material

Syrup called Bronchosol (Phytopharm, Kleka, Poland) available on the Polish market was used in the study. Hundred milliliters of Bronchosol contain the following active ingredients: 4.36 g of a dense extract consisting of (3 : 1; aq.) thyme and primula root (7.6 : 1) and 19.8 g of thymol (concentration for use). The excipients include orange flavor, sugar and purified water.

The antimicrobial activity of Bronchosol was determined on the following 20 strains of anaerobic bacteria isolated from patients with respiratory infections: *Peptostreptococcus anaerobius* (2 strains), *Finegoldia magna* (2 strains), *Micromonas micros* (2 strains), *Actinomyces odontolyticus* (1 strain), *Bifidobacterium breve* (1 strain), *Propionibacterium acnes* (1 strain), *Propionibacterium granulosum* (1 strain), *Prevotella intermedia* (3 strains), *Prevotella levii* (1 strains), *Prevotella loescheii* (1 strain), *Porphyromonas asaccharolytica* (1 strain), *Porphyromonas gingivalis* (1 strain), *Fusobacterium nucleatum* (1 strain), *Fusobacterium necrophorum* (1 strain), *Bacteroides fragilis* (1 strain) and 6 reference strains: *P. anaerobius*, *F. magna*, *P. acnes*, *B. breve*, *F. nucleatum*, *B. fragilis*.

As far as the aerobic bacteria are concerned, 13 strains (one from each species) were isolated from materials obtained from patients. The species included: *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Streptococcus anginosus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, *Citrobacter freundii*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*. There were 7 reference strains, namely *S. aureus*, *S. pneumoniae*, *S. pyogenes*, *H. influenzae*, *Moraxella catarrhalis*, *K. pneumoniae*, *P. aeruginosa*. Five strains of yeast-like fungi isolated from patients were also subjected to the study and these belonged to the following species: *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*. There were 5 reference strains, too.

The materials collected from patients were inoculated on selective and non-selective media

appropriate for the given microorganisms, which were incubated in anaerobic jar or aerobic conditions for the right period of time. The isolated strains of anaerobic (31-34) and aerobic (33-35) bacteria as well as the fungi (36, 37) were identified in accordance with the present principles.

Methods

Antibacterial activity

Determination of minimal bactericidal concentration

In order to determine the minimal bactericidal concentration (MBC) of Bronchosol, 0.1 mL of a suspension of the anaerobic or aerobic bacterial strains, containing 10^6 CFU (colony forming units) / mL was added to 1 mL of the (undiluted) preparation. Next, after 15 and 30 min, 0.1 mL was taken and inoculated into 2 mL of thioglycolate broth (anaerobic bacteria) or Brain Hearth Infusion (BHI) broth (Merck) (aerobic bacteria). The medium inoculated with 0.1 mL of the appropriate bacteria culture was used to control the growth of a given strain. The incubation of the tested and control media was conducted at 37°C for 48 h in anaerobic jars (in anaerobic conditions) for the anaerobic bacteria, and at 37°C for 24 h in aerobic conditions for the aerobic bacteria. A lack of any bacteria growth in the medium after appropriately long incubation proved bactericidal activity (MBC) of the preparation.

Determination of minimal inhibitory concentration

The minimal inhibitory concentration (MIC) of the bacterial strains was determined by the method of

Table 1. Bactericidal activity (MBC - minimal bactericidal concentration) of Bronchosol at concentration recommended by producer against reference strains of anaerobic bacteria and bacteria isolated from patients with respiratory infections.

Anaerobic bacteria	Number of strains tested	Number of strains sensitive to preparation	
		after 15 min	after 30 min
Gram-positive cocci:			
*Peptostreptococcus anaerobius**	2	2	2
*Finegoldia magna**	2	2	2
*Micromonas micros**	2	2	2
Total Gram-positive cocci	**6**	**6**	**6**
Gram-positive baccilli:			
*Actinomyces odontolyticus**	1	1	1
Bifidobacterium breve	1	1	1
*Propionibacterium acnes**	1	0	1
*Propionibacterium granulosum**	1	0	1
Total Gram-positive baccilli	**4**	**2**	**4**
Total Gram-positive anaerobic bacteria	**10**	**8**	**10**
Gram-negative baccilli:			
*Prevotella intermedia**	3	3	3
*Prevotella levii**	1	1	1
*Prevotella loescheii**	1	1	1
*Porphyromonas asaccharolytica**	1	1	1
*Porphyromonas gingivalis**	1	1	1
*Fusobacterium nucleatum**	1	1	1
*Fusobacterium necrophorum**	1	0	1
*Bacteroides fragilis**	1	0	0
Total Gram-negative baccilli	**10**	**8**	**9**
Standard strains			
Finegoldia magna ATCC 29328	1	1	1
Peptostreptococcus anaerobius ATCC 27337	1	1	1
Propionibacterium acnes ATCC 11827	1	1	1
Bifidobacterium breve ATCC 15700	1	1	1
Fusobacterium nucleatum ATCC 25586	1	1	1
Bacteroides fragilis ATCC 25585	1	0	0
Total standard strains	**6**	**5**	**5**
Total anaerobic bacteria	**26**	**21**	**24**

* - anaerobic bacteria strains isolated from patients with respiratory infections, 0 - no effect of the preparation.

serial dilution of Bronchosol on the right medium. In the case of the anaerobic bacteria, Brucella agar with 5% of defibrinated sheep blood, menadione and hemin was used, while Mueller-Hinton agar was used for the aerobic bacteria. The following concentrations of the preparation were tested: 6.2, 12.5, 25.0, 50.0, 100.0 and 200.0 mg/mL. A suspension containing 10^6 CFU per drop was placed on the surface of the right agar (depending on the bacteria type) with Steers replicator, after previous addition of the appropriate concentration of the preparation to the agar. Agar which did not contain Bronchosol was used to control the strain growth. The inoculated media and the control media were incubated in either anaerobic (in anaerobic jars) or aerobic conditions, at 37°C for the right period of time depending on the bacteria type: for 48 h in the case of the anaerobic bacteria and for 24 h in the case of the aerobic bacteria. The MIC was defined as the lowest concentrations of the preparation that completely inhibited the growth of the tested anaerobic or aerobic bacteria strains.

The yeast-like fungi strains cultured from the materials obtained from patients with infections of the upper respiratory tract were inoculated in Sabouraud's agar and incubated at 37°C for 24-72 h.

Antifungal activity

Determination of minimal fungicidal concentration

In order to assess the fungicidal activity (MFC - minimal fungicidal concentration), 0.1 mL of a suspension of the culture of the tested strain containing 10^6 CFU in 1 mL was added to 1 mL of Bronchosol (undiluted preparation). After 15 and 30 min, samples of 0.1 mL were taken and inoculated into 2 mL of BHI broth (Merck). The BHI broth inoculated with 0.1 mL of the fungal culture constituted the growth control of a given strain. The inoculated and control media were incubated at 37°C for 24 h in aerobic conditions. A lack of any yeast-like fungi growth in the medium proved fungicidal activity of the preparation.

Table 2. Bactericidal activity (MBC - minimal bactericidal concentration) of Bronchosol at concentration recommended by producer against reference strains of aerobic bacteria and bacteria isolated from patients with respiratory infections.

Aerobic bacteria	Number of strains tested	Number of strains sensitive to preparation	
		after 15 min	after 30 min
Gram-positive cocci:			
*Staphylococcus aureus**	1	1	1
*Staphylococcus epidermidis**	1	1	1
*Enterococcus faecalis**	1	0	0
*Streptococcus anginosus**	1	1	1
*Streptococcus pneumoniae**	1	1	1
*Streptococcus pyogenes**	1	1	1
Total Gram-positive cocci	**6**	**5**	**5**
Gram-negative baccilli:			
*Acinetobacter baumannii**	1	1	1
*Citrobacter freundii**	1	0	1
*Escherichia coli**	1	1	1
*Haemophilus influenzae**	1	0	1
*Klebsiella pneumoniae**	1	0	1
*Pseudomonas aeruginosa**	1	0	0
*Serratia marcescens**	1	1	1
Total Gram-negative baccilli	**7**	**3**	**6**
Standard strains:			
Staphylococcus aureus ATCC 25923	1	1	1
Streptococcus pneumoniae ATCC 49619	1	1	1
Streptococcus pyogenes ATCC 19615	1	1	1
Haemophilus influenzae ATCC 49274	1	1	1
Moraxella catarrhalis ATCC 25238	1	1	1
Klebsiella pneumoniae ATCC 13883	1	0	1
Pseudomonas aeruginosa ATCC 27853	1	0	0
Total standard strains	**7**	**5**	**6**
Total aerobic bacteria	**20**	**13**	**17**

* - aerobic bacteria strains isolated from patients with respiratory infections, 0 - no effect of the preparation.

Table 3. Sensitivity (MIC - minimal inhibitory concentration) to Bronchosol of anaerobic bacteria isolated from patients with respiratory infections and that of reference strains.

Anaerobic bacteria	Number of strains tested	MIC [mg/mL]					
		≥ 200.0	100.0	50.0	25.0	12.5	6.2
Gram-positive cocci:							
*Peptostreptococcus anaerobius**	2	1	1	0	0	0	0
*Finegoldia magna**	2	1	1	0	0	0	0
*Micromonas micros**	2	1	1	0	0	0	0
Total Gram-positive cocc	**i 6**	**3**	**3**	**0**	**0**	**0**	**0**
Gram-positive baccilli:							
*Actinomyces odontolyticus**	1	0	1	0	0	0	0
*Bifidobacterium breve**	1	0	1	0	0	0	0
*Propionibacterium acnes**	1	1	0	0	0	0	0
*Propionibacterium granulosum**	1	1	0	0	0	0	01
Total Gram-positive baccilli	**4**	**2**	**2**	**0**	**0**	**0**	**0**
Gram-negative baccilli:							
*Prevotella intermedia**	3	3	0	0	0	0	0
*Prevotella levii**	1	1	0	0	0	0	0
*Prevotella loescheii**	1	1	0	0	0	0	0
*Porphyromonas asaccharolytica**	1	1	0	0	0	0	0
*Porphyromonas gingivalis**	1	1	0	0	0	0	0
*Fusobacterium nucleatum**	1	1	0	0	0	0	0
*Fusobacterium necrophorum**	1	1	0	0	0	0	0
*Bacteroides fragilis**	1	1	0	0	0	0	0
Total Gram-negative baccilli	**10**	**10**	**0**	**0**	**0**	**0**	**0**
Standard strains:							
Finegoldia magna ATCC 29328	1	0	1	0	0	0	0
Peptostreptococcua anaerobius ATCC 27337	1	1	0	0	0	0	0
Propionibacterium acnes ATCC 11827	1	1	0	0	0	0	0
Bifidobacterium breve ATCC 25286	1	1	0	0	0	0	0
Fusobacterium nucleatum ATCC 25586	1	1	0	0	0	0	0
Bacteroides fragilis ATCC 25285	1	1	0	0	0	0	0
Total standard strains	**6**	**5**	**1**	**0**	**0**	**0**	**0**
Total anaerobic bacteria	**26**	**20**	**6**	**0**	**0**	**0**	**0**

* - anaerobic bacteria strains isolated from patients with respiratory infections, 0 - no effect of the preparation.

The sensitivity test (MIC) of the yeast-like fungi to Bronchosol was conducted by the method of serial dilution in Sabouraud's agar (38). The following dilutions of the preparation were tested: 6.2, 12.5, 25.0, 50.0, 100.0 mg/mL. An inoculum containing 10^6 CFU per drop was placed on the surface of the agar with Steers replicator. A medium without the preparation was used to control the growth of the tested fungi strains. Incubation of the inoculated and control media was performed at 37^{o}C for 24 h in aerobic conditions. The MIC was defined as the lowest concentrations of Bronchosol which completely inhibited the growth of the tested strains of yeast-like fungi.

RESULTS AND DISCUSSION

The aim of the study was to determine the antimicrobial activity of Bronchosol (Phytopharm, Klęka) against anaerobic and aerobic bacteria, and fungi that cause respiratory infections most frequently, isolated from patients with respiratory infections, and reference ones.

The study was conducted in *in vitro* conditions slightly different from *in vivo* conditions. All tests were performed at a temperature of 37^{o}C, similar to the temperature found in the oral cavity and upper respiratory tract of a healthy organism. A bacterial, viral or fungal infection usually causes a rise in the temperature, which can be controlled with the use of antipyretics. The neutral pH found in the oral cavity and upper respiratory tract is similar to the pH reaction of the applied media (6.4 – 7.4), except for Sabouraud's agar – pH 5.4, whose acidic reaction corresponds to pH of the oral cavity during and after a meal. The exposure of the microorganisms to Bronchosol in the experimental conditions lasted 48

Table 4. Sensitivity (MIC - minimal inhibitory concentration) to Bronchosol of aerobic bacteria isolated from patients with respiratory infections and that of reference strains.

Aerobic bacteria	Number of strains tested	MIC [mg/mL]					
		≥ 200.0	100.0	50.0	25.0	12.5	6.2
Gram-positive cocci:							
*Staphylococcus aureus**	1	0	0	1	0	0	0
*Staphylococcus epidermidis**	1	0	0	0	0	0	1
*Enterococcus faecalis**	1	1	0	0	0	0	0
*Streptococcus anginosus**	1	0	1	0	0	0	0
*Streptococcus pneumoniae**	1	0	0	1	0	0	0
*Streptococcus pyogenes**	1	0	0	0	0	1	0
Total Gram-positive cocci	**6**	**1**	**1**	**2**	**0**	**1**	**1**
Gram-negative baccilli:							
Acinetobacter baumannii *	1	0	1	0	0	0	0
*Citrobacter freundii**	1	1	0	0	0	0	0
*Escherichia coli**	1	1	0	0	0	0	0
*Haemophilus influenzae**	1	0	0	1	0	0	0
*Klebsiella pneumoniae**	1	1	0	0	0	0	0
*Pseudomonas aeruginosa**	1	1	0	0	0	0	0
*Serratia marcescens**	1	1	0	0	0	0	0
Total Gram-negative baccilli	**7**	**5**	**1**	**1**	**0**	**0**	**0**
Standard strains:							
Staphylococcus aureus ATCC 25923	1	0	1	0	0	0	0
Streptococcus pneumoniae ATCC 49619	1	1	0	0	0	0	0
Streptococcus pyogenes ATCC 19615	1	0	0	0	0	1	0
Haemophilus influenzae ATCC 49247	1	0	0	1	0	0	0
Moraxella catarrhalis ATCC 25238	1	1	0	0	0	0	0
Klebsiella pneumoniae ATCC 13883	1	1	0	0	0	0	0
Pseudomonas aeruginosa ATCC 27853	1	1	0	0	0	0	0
Total standard strains	**7**	**4**	**1**	**1**	**0**	**1**	**0**
Total aerobic bacteria	**20**	**10**	**3**	**4**	**0**	**2**	**1**

* - aerobic bacteria strains isolated from patients with respiratory infections, 0 - no effect of the preparation.

Table 5. Fungicidal activity (MFC - minimal fungicidal concentration) of Bronchosol at concentration recommended by producer against reference strains of yeast-like fungi and strains isolated from patients with respiratory infections.

Yeast-like fungi	Number of strains tested	Number of strains sensitive to preparation	
		after 15 min	after 30 min
*Candida albicans**	3	3	3
*Gandida glabrata**	1	1	1
*Candida krusei**	1	1	1
*Candida parapsilosis**	1	1	1
*Candida tropicalis**	1	1	1
Total yeast-like fungi	**7**	**7**	**7**
Standard strains:			
Candida albicans ATCC 10231	1	1	1
Candida glabrata ATCC 66032	1	1	1
Candida krusei ATCC 14234	1	1	1
Candida parapsilosis ATCC 22019	1	1	1
Candida tropicalis ATCC 750	1	1	1
Total standard strains	**5**	**5**	**5**
Total yeast-like fungi	**12**	**12**	**12**

* - yeast-like fungi strains isolated from patients with respiratory infections.

Table 6. Sensitivity (MIC - minimal inhibitory concentration) to Bronchosol of yeast-like fungi from genus *Candida* isolated from patients with respiratory infections and that of reference strains.

Yeast-like fungi	Number of strains tested	MIC [mg/mL]					
		≥ 200.0	100.0	50.0	25.0	12.5	6.2
*Candida albicans**	3	1	1	1	0	0	0
*Candida glabrata**	1	0	1	0	0	0	0
*Candida krusei**	1	1	0	0	0	0	0
*Candida parapsilisis**	1	0	1	0	0	0	0
*Candida tropicalis**	1	0	1	0	0	0	0
Total yeast-like fungi	**7**	**2**	**4**	**1**	**0**	**0**	**0**
Standard strains:							
Candida albicans ATCC 10231	1	0	1	0	0	0	0
Candida glabrata ATCC 66032	1	1	0	0	0	0	0
Candida krusei ATCC 14234	1	1	0	0	0	0	0
Candida parapsilosis ATCC 22019	1	0	1	0	0	0	0
Candida tropicalis ATCC 750	1	0	1	0	0	0	0
Total standard strains	**5**	**2**	**3**	**0**	**0**	**0**	**0**
Total yeast-like fungi	**12**	**4**	**7**	**1**	**0**	**0**	**0**

* - yeast-like fungi strains isolated from patients with respiratory infections, 0 - no effect of the preparation.

and 24 h in the case of the anaerobic and aerobic bacteria, respectively, and between 24 and 72 h for the fungi. In *in vivo* conditions, the exposure of the microorganisms to the ingredients of the preparation was initially limited to several-minute local activity of the syrup, which covers the mucosa of the oral cavity and throat; however, after absorption of the active ingredients by the mucosa of the oral cavity and throat as well as from the digestive tract, the active ingredients get with blood to the lower and upper respiratory tract, where they show antimicrobial activity.

The majority of the strains used in the study were isolated from material obtained from patients with respiratory infections; 13 of them were reference strains.

The preparation showed great bactericidal activity (concentration for use) against all 46 bacterial strains tested. In the case of the anaerobic bacteria, 15 min after application of Bronchosol, 80% of the Gram-positive bacteria were killed, including 60% of the cocci, and after 30 min all the tested strains of these bacteria were killed. Similar bactericidal activity was observed against the Gram-negative anaerobic baccilli, 80% of which were killed after 15 min and 90% after 30 min after application of Bronchosol. It is worth pointing out that after 15 min, the preparation showed bactericidal activity against 80% of all the anaerobic bacteria tested, and after 30 min – against 95% of these bacteria (Table 1). In the case of the aerobic bacteria, Bronchosol showed great activity, only 15 min after application, against the Gram-positive cocci (83%), including the bacteria strains which participate in respiratory infections more frequently, i.e., *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Streptococcus anginosus*. This activity did not change after 30 min since the application of the preparation. The Gram-negative baccilli, however, were less susceptible to the activity of Bronchosol. After 15 min of its activity, 43% of the strains were killed, but the biocidal activity increased 30 min after the application of the preparation and affected 86% of the strains, including those from the following species: *Acinetobacter baumannii*, *Escherichia coli*, *Haemophilus influenzae* and *Klebsiella pneumonia* (Table 2).

The results of the study in sensitivity (MIC) (Table 3) suggested that Bronchosol ranging between ≤ 6.2 – 100.0 mg/mL inhibited growth of the Gram-positive anaerobic bacteria, including half of the investigated cocci. The activity against the Gram-negative baccilli was lower (MIC ≥ 200.0 mg/mL). The aerobic bacteria tested, on the other hand, demonstrated greater sensitivity to the preparation in question (Table 4). Growth of the Gram-positive cocci was inhibited when concentration ranged between ≤ 6.2 – 100.0 mg/mL (83%). The greatest sensitivity was shown, among others, by strains from the genus *Streptococcus pyogenes*, which often participate in infections of the respiratory tract (MIC ≤ 6.2 – 12.5 mg/mL). The strains of the Gram-negative baccilli turned out to be less sensitive. Out of the 7 tested strains, only 2 (29%) showed sensitivity when the concentration ranged

from 50.0 to 100.0 mg/mL. Growth of the other strains was inhibited when concentration was ≥ 200.0 mg/mL. The greatest sensitivity among the Gram-negative baccilli was displayed by strains from the species *Acinetobacter baumannii* and *Haemophilus influenzae* (MIC ranging from 50.0 to 100.0 mg/mL). High fungicidal activity of Bronchosol against all the tested fungi from the genus *Candida* was also observed. All the investigated strains were killed 15 min after application of the preparation (Table 5). Five (71%) strains of the tested yeast-like fungi from the genera *Candida* were sensitive (MIC) to concentration ranging from 50.0 to 100.0 mg/mL. To inhibit growth of the other strains (29%), higher concentration of Bronchosol, more than 200.0 mg/mL, was required (Table 6).

The strong activity against the abovementioned pathogens probably resulted from synergism between the compounds of thyme, especially the components of the essential oil, primula root and thymol. It should be emphasised that some of the bacteria and fungi were highly sensitive to Bronchosol and, therefore, the antibacterial and antifungal effects were achieved with concentrations several times lower than the ones usually applied. The bacterial strains, frequently participating in infections and demonstrating resistance to medicines, i.e., for example, *Staphylococcus aureus* or the fungi *Candida glabrata*, *Candida krusei*, *Candida tropicalis*, turned out to be sensitive to the activity of Bronchosol.

CONCLUSION

Application of multicomponent preparations enables to obtain a stronger desired effect at lower concentrations of substances and extracts, and decreases the risk of drug resistance of bacteria. The activity against fungi from the genus *Candida* is especially beneficial in the treatment of respiratory infections. Antibiotic therapy leads to destruction of physiological bacterial flora and occurrence of the right conditions for the development of candidosis. The antifungal activity of Bronchosol may prevent candidosis in patients treated with antibiotics.

Thus, Bronchosol is a syrup recommended not only to treat the symptoms of respiratory infections (a cough, impeded expectoration of lingering phlegm), but, more importantly in light of the aforementioned results, it may also be effective as a basic/primary drug affecting the cause of the illness – bacteria and fungi, and as a supporting preparation in an antibiotic therapy.

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PERCEPTION OF ACADEMIC PHARMACISTS TOWARDS THEIR ROLE IN HEALTHCARE SYSTEM OF A DEVELOPING COUNTRY, PAKISTAN: A QUANTITATIVE SIGHT

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Abstract: To investigate perception of academic pharmacists towards their role in healthcare system of a developing country, Pakistan, was the aim of this study. The study participants consisted of academic pharmacists from the government and private universities of Pakistan. Study was conducted for a period of three months, from January to March 2011, in Pakistan. Academic pharmacists were informed regarding the aim, objective and nature of the study. Verbal consent was given and execution of the study took place. Main sections of the questionnaire were comprised of series of statements pertaining to pharmacist perception and experience with the pharmacists. During the period, of January to April, the total number of questionnaires received from different government and private universities was 113. The response rate based on the 205 academic pharmacists working in universities during the study period was 63.9%. A majority (93.9%) of the respondents were satisfied working as academic pharmacist, 68.7% expressed that their interest in research is the reason to embark carrier as an academic pharmacist. Only half (55.7%) of the respondents agreed about pharmacy curriculum standard in Pakistan, and their satisfaction towards curriculum is less, as after the implementation of Pharm. D. (Doctor of Pharmacy). In the country, the curriculum is still focusing more towards theoretical knowledge rather than practical. In this case significant difference was noted with respect to university ($p = 0.012$). Academic pharmacists do have concern about the pharmacy profession in Pakistan. They ascertain the key issues facing the profession and its educators. Recommendations have been made to improve the current pharmacy curricula in order that future pharmacists in Pakistan practice effectively with the collaboration of other healthcare professionals.

Keywords: academic pharmacist, perception, pharmacist role, pharmacy curriculum, face-lifting.

A pharmacist is the healthcare professional who has responsibility of ensuring the rational use of medicines. Pharmacists play a vital part in the delivery of healthcare world-wide. There is an increasing number of opportunities for pharmacists to assume a greater role in helping patients make better use of their medications and achieve optimal therapeutic outcomes in both the public and private sector (1). World Health Organization (WHO) and International Pharmaceutical Federation (FIP) recommended that the basic role of the pharmacist should cover seven major components: care-giver, decision-maker, communicator, leader, manager, life-long-learner, and finally teacher. WHO also mandated that future pharmacists must possess specific knowledge, attitude, skill and behavior in order to support their roles (2).

Academic pharmacist, like in the other pharmacy fields is facing the acute man-power shortage (3, 4). In many countries, workforce shortage also applies to academia, hence, the capacity to scale up education may be limited (5). Effects made to improve the shortage of pharmacists and to some extent these effects have improved the situation slowly, but the situation in academia is still poor (6). One of the major factor which is commonly faced by

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the developed and developing countries is the increasing enrollments of students within schools and colleges of pharmacy (7-9). Such expansion presents many concerns, including its effect on the quality of teaching, the number of available pharmacy-trained academic faculty members and the academic standard of applicants, which have not been matched by similar increases in resources, including staffing levels (10).

There are studies which focused on the pharmacy education in both developed and developing countries, but very few studies investigated the academic pharmacists' perceptions on their role in healthcare system. Although a few studies have been conducted in countries such as United States and Canada addressing this issue, it is very difficult to generalize these foreign studies into Pakistani context. Thus, the aim of this study was to investigate perception of academic pharmacists towards their role in healthcare system of Pakistan.

METHODOLOGY

The questionnaire was developed from the extensive review of literature and on the basis of the phase 1 of that study, that is, qualitative part. The questionnaire had four sections on demographic information, choice for career as an academic pharmacist, perception regarding current pharmacy curriculum and opinions about Pakistan Pharmacist Association PPA.

Section 3 of the questionnaire included a set of statements for which the respondents were asked to indicate the level of agreement using a 4-point Likert scale, whereby 1 = strongly disagreed, 2 = disagreed, 3 = agreed, 4 = strongly agreed. A four Likert scale was used in order to avoid confusion with the 'neutral' responses.

Table 1. Academic pharmacists from universities.

Universities	Number of questionnaires received
University 1	19
University 2	14
University 3	13
University 4	13
University 5	12
University 6	10
University 7	8
University 8	18
University 9	14
University 10	10

Face and content validation of questionnaire

To assess validity of content, the preliminary version of the questionnaire consisting of 17 items was sent to the professionals at the School of Pharmacy, Universiti Sains Malaysia. These professionals were asked to assess the questionnaire by providing their overall opinions and by listing the questions in the order of relevance and importance. The questions of more relevance and importance were highlighted.

To assess face validity of the questionnaire, copies of the questionnaire were distributed among 20 participants. These participants were asked for their views on the significance, worth, and simplicity of each question and to identify as to which questions they would point out 'need to be removed' so as to make the questionnaire brief. In addition to this, the participants were also welcomed to suggest further comments on the questions whether they are understandable or not. Most of them suggested simplifying the questions. The reliability test was applied to all the variables comprising the knowledge domain. The reliability of the tool was estimated on the basis of Cronbach's Alpha ($\alpha = 0.617$).

Study participants and sample size

The study participants consisted of academic pharmacists from the government and private universities of Pakistan. The number of questionnaires received from the universities is given in Table 1.

Survey administration and time frame

The survey was conducted for a period of three months, from January to March 2011, in Pakistan. Academic pharmacists were informed regarding the aim, objective and nature of the study. Verbal consent was given and execution of the study took place.

Data collection

The questionnaires were collected from the academic pharmacists. Responses were exported to Statistical Package for Social Sciences (SPSS®) for Windows, version 15, to perform statistical analysis (11).

Data analysis

Non-parametric statistical test and appropriate descriptive statistics for demographic characteristics (mean and standard deviation for age) were performed using SPSS® for Windows, version 15. The

demographic information that was collected included: age, gender, type of university, years of experience and current post. Frequencies and descriptive statistic of each variable was reported and the mean and standard deviation were calculated for age. The Fisher's exact test was used to test the significance of association between the independent variables (age, gender, type of university, and years of experience) and the dependent variables (respondents, perception regarding current pharmacy curriculum).

Table 2. Academic pharmacist from universities.

Universities	Number of academic pharmacists	Number of questionnaires received	Percentage of pharmacists responded (%)
University 1	21	19	90.4
University 2	26	14	53.8
University 3	25	13	52
University 4	15	13	86.6
University 5	15	12	80
University 6	17	10	58.8
University 7	13	8	61.5
University 8	23	18	78.2
University 9	18	14	77.7
University 10	32	10	31.25
Total	205	131	63.9

Table 3. Academic pharmacists' demographic characteristics.

Variables	Frequency	Percent
Age (mean ± SD, 34.02 ± 7.9)		
20-30	57	43.5
31-40	49	37.4
41-50	20	15.3
>50	5	3.8
Gender		
Male	83	63.4
Female	48	36.6
Type of university		
Public	81	61.8
Private	50	38.2
Years of experience		
1-10	110	84.0
11-20	19	14.5
21-30	2	1.5
Current post		
Demonstrator	18	13.7
Lecturer	75	57.3
Assistant professor	31	23.7
Associate professor	3	2.3
Professor	4	3.1

Fisher's exact test was used because it is considered to be more appropriate for skewed data, moreover, as rule of thumb, if 25% or more of the cells in the table have expected frequencies less than 5, or if any expected frequency is less than 1, then Fisher's exact p-value is computed. A p-value of 0.05 or less was considered to be statistically significant.

RESULTS AND DISCUSSION

Response rate

During the period, of January to April, the total number of questionnaires received from different government and private universities was 113. The response rate, based on the 205 academic pharmacists working in universities during the study period, was 63.9%. Response rate according to universities are shown in Table 2.

Part 1 - Demographic

Demographic characteristics of respondents from each university are shown in Table 3. The average age of the respondents was 34.02 years, standard deviation = 7.9. Among the respondents 63.4% were male, whereas 36.6% were female. A majority of 61.8% respondents were from public universities. With respect to years of teaching experience, 84.0% of the respondents had 1 to 10 years of experience. Among the academic pharmacists, 13.7% were demonstrators, 57.3% were lecturers, 23.7% were assistant professors, 2.3% were associate professors, and 3.1% were professors.

Part 2 - Choice of academic career

Respondents were asked about their choice to be academic pharmacists in Table 4. A majority (93.9%) of the pharmacists were satisfied with their work and 82.4% took academia as their first choice career. Indicating the reasons for joining, 68.7% showed their interest in research, 74.8% showed interest for higher education. Only 27.5% of the pharmacists indicated the lack of opportunities in other sectors. A majority of the pharmacists, 88.5%, would go for academic pharmacy as a profession, if they were given another choice for career.

Part 3 - Perception regarding current pharmacy curriculum

Perceptions of academic pharmacists regarding pharmacy curriculum are shown in Table 5. Only 55.7% of the academic pharmacists agreed when they were asked about the pharmacy curriculum that it is up to the defined standard. The value was found to be of statistical significance ($p = 0.009$) with respect to type of university. With reference to the pharmacy curriculum which covers the clinical aspect of the pharmacy, 53.4% of the pharmacists accepted it and the value is of statistical significance ($p = 0.002$) with the type of university. When they were asked about their satisfaction with the pharmacy curriculum at undergraduate level, 61.8% of the respondents agreed. With respect to distribution of both theoretical and practical aspect, 48.1% of the pharmacists disagreed. Half of the pharmacists, 53.4%, agreed on the current introduction of 5 years Pharm. D. program in all the Pakistani pharmacy schools, which will help to develop better practitioners; with respect to gender, the p value was found to be statistically significant ($p = 0.044$).

Part 4 - View regarding Pakistan Pharmacist Association

Table 6 indicates the views about PPA. A majority (89.3%) of the academic pharmacists knows about the representative body of Pharmacists in Pakistan. Only 39.7% held the membership of

Table 4. Choice for career as academic pharmacist.

Items in questionnaire	Frequency	Percent
Are you satisfied working as an academic pharmacist?	123	93.9
Was joining as academic pharmacist is your first choice of carrier?	108	82.4
Interest in research	90	68.7
Interest for higher education	98	74.8
Lack of opportunities in other sectors	36	27.5
If you were given another carrier choice again, would you go for academic pharmacy as profession	116	88.5

Table 5. Perception regarding current pharmacy curriculum.

Items in questionnaire	Responses*				p-value**			
	SD (n, %)	D (n, %)	A (n, %)	DA (n, %)	Age	Gender	Type of university	Year of experience
1	7(5.3)	49(37.4)	73(55.7)	2(1.5)	0.060	0.291	**0.009**	0.494
2	12(9.2)	48(36.6)	70(53.4)	1(0.8)	0.922	0.609	**0.002**	0.958
3	5(3.8)	56(42.7)	67(51.1)	3(2.3)	0.134	0.555	**0.023**	0.483
4	5(3.8)	41(31.3)	81(61.8)	4(3.1)	0.100	0.081	0.715	0.357
5	3(2.3)	63(48.1)	60(45.8)	5(3.8)	0.382	0.321	0.508	0.245
6	7(5.3)	42(32.1)	70(53.4)	12(9.2)	0.172	**0.044**	0.148	0.459

*Reported responses were for all respondents; ** Fisher's Exact test; 1 = Pharmacy curriculum is up to the define standard; 2 = Curriculum covers all the aspect of pharmacy practice; 3 = Curriculum also covers the clinical aspect of the pharmacy; 4 = Are you satisfied with the pharmacy curriculum at undergraduate level ? 5 = Are you satisfied with the distribution of both theoretical and practical aspect? 6 = Current introduction of 5 years Pharm. D. program in all the Pakistani pharmacy schools, will help to develop better practitioners.

Table 6. Views regarding Pakistan Pharmacists Association.

Items in questionnaire	Frequency	Percent
1	117	89.3
2	52	39.7
3	67	51.1
4	69	52.7

1 = Do you know about the representative body of Pharmacists in Pakistan (PPA)? 2 = Do you currently hold membership of the PPA? 3 = Have you ever participated in any event held by the PPA? = PPA is the representative body for pharmacist working for the interest of pharmacy profession.

PPA. More than a half of the academic pharmacists (52.7%) believed that PPA is one of the representative body of pharmacists working for the interest of pharmacy profession.

Academic pharmacy is facing man power shortage (3, 6) not only in developed countries but also in developing countries. In order to overcome the shortage, a number of efforts were made which, in turn, improved the situation better than before. Situation in Pakistan is not much different as compared to other parts of the world; pharmacy profession is facing acute storage in the country. In case of academia, only 5% of the pharmacists are working as academic pharmacists (12). The finding of the present study shows that the academic pharmacists working are highly satisfied with their career. The finding is consistent with the study in Lebanon (13), where the pharmacists are recruited as academic pharmacists and they never change their career; they are highly satisfied with respect to financial, psychological and physical aspects. Interest in higher education is one of the major reasons of joining as an academic pharmacist. In 2003, the doctor of pharmacy (Pharm. D.) started in Pakistan and was focused on the clinical aspects of the pharmacy profession, and due to it the change in the curriculum appeared. The present study focused on the perception of academic pharmacists regarding curriculum; only half of the respondents agreed with the pharmacy curriculum. The findings are consistent with the letter which explains the issues raised with the start of Pharm. D. in Pakistan with respect to pharmacy curriculum in terms of lack of experienced academicians and practice based facilities (14, 15). With the distribution of theoretical and practical aspects of the curriculum, the pharmacists are not satisfied and this issue was highlighted in the review which discussed that the pharmacist's role was yet to be recognized in Pakistan (16). One of the reasons for the pharmacists' lack of recognition by other health professionals is the lack of practical exposure, as pharmacy institutions in Pakistan exist without an attached hospital where pharmacy students can acquire basic clinical knowledge and that was noticed in other developing countries too (17). In contrast, Arab countries like Kuwait, Saudi

Arabia and Jordan have already introduced clinical placements which are not only related to pharmacy but also to medical rotations (9, 18, 19). Academic pharmacists know about the PPA as one of the representing body of pharmacists in Pakistan. Unfortunately, only half of the respondents hold its membership. They believed that PPA is working for the interest of the pharmacists in the country.

One of the major limitations of the study is the winter vacation in the two provinces of the country during the time of data collection. Most of the academic pharmacists cannot be reached as they left for higher education abroad. Time and financial constraints are another contributing factors for low response rate.

CONCLUSION

The pharmacists play a critical role in improving the healthcare in the society, which can only be achieved by providing the adequate learning opportunities for the students, and which will be the future of practicing pharmacists. Based on the findings of the survey, there is a lack of practical exposure for the pharmacy students. There is a strong and uniform desire in both the public and private academia that the major emphasis should be placed on the improvement and amendments in terms of pharmacy curriculum. For the uplifting of pharmacy profession in Pakistan, support of government and pharmacy organization is very essential. Then, the pharmacy practice will be as advanced as it is in other parts of the world.

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MICROBIOLOGICAL QUALITY OF FOOD SUPPLEMENTS

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Abstract: Many specialists note that the food offered today – as a result of very complex technological processing – is devoid of many components that are important for the organism and the shortages have to be supplemented. The simplest for it is to consume diet supplements that provide the missing element in a concentrated form. In accordance with the applicable law, medicinal products include all substances or mixtures of substances that are attributed with properties of preventing or treating diseases with humans or animals. Permits to admit supplements to the market are issued by the Chief Sanitary Inspector and the related authorities; permits for medicines are issued by the Chief Pharmaceutical Inspector and the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products. Therefore, admittance of a supplement to the market is less costly and time consuming than admittance of a medicine. Supplements and medicines may contain the same component but medicines will have a larger concentration than supplements. Sale of supplements at drug stores and in the form of tablets, capsules, liquids or powders makes consumer often confusing supplements with medicines. Now there are no normative documents specifying limits of microbiological impurities in diet supplements. In Polish legislation, diet supplements are subject to legal acts concerning food. Medicines have to comply with microbiological purity requirements specified in the Polish Pharmacopeia. As evidenced with the completed tests, the proportion of diet supplement samples with microbiological impurities is 6.5%. Sales of diet supplements have been growing each year, they are consumed by healthy people but also people with immunology deficiencies and by children and therefore consumers must be certain that they buy safe products.

Keywords: microbiological purity, Good Manufacturing Practice (GMP), diet supplements

According to the definition included in the Act of 25 August 2006 on the Safety of Food and Nutrition, dietary supplements are "foodstuffs intended to add further nutritional value to the normal diet, representing concentrated sources of vitamins, minerals or other substances having a nutritional or otherwise physiological effect, either individual or combined, marketed in a dosage form" (1).

There are currently no normative documents specifying microbiological contamination limits for dietary supplements. In the Polish legislation, dietary supplements are subject to legal acts regulating food products. Pursuant to the Act on the Safety of Food and Nutrition, all business entities operating on the food market are obliged to comply with requirements of Good Hygiene Practice (GHP) in their production plants, and follow rules embodied in the Hazard Analysis and Critical Control Points (HACCP) system.

The aim of this work was to retrospectively analyze results of studies investigating microbiological purity of dietary supplements produced in pharmaceutical manufacturing plants in the Wielkopolska region.

MATERIALS AND METHODS

The analysis comprised results of microbiological purity tests performed for a total of 1165 samples of the dietary supplements in form of tablets. Tablets were the final forms of dietary supplements in the sales package, from three manufacturing plants. The study was performed within one month from the date of manufacture of the product. The studies were conducted over a period of three years (from 2010 to 2012). Testing performed according to requirements set out by the ordering party involved determination of total aerobic count, total fungal count, presence and count of Gram-negative *Enterobacteriaceae* bacteria, presence of *Pseudomonas aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* in 1 g/mL, and *Salmonella* in 10

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g/mL. Microbiological testing of preparations was carried out in compliance with methods laid down in relevant Polish Pharmacopoeia (PP) monographs (2). For this purpose, 10 gram of product to be examined was dissolved in 100 mL of buffered sodium chloride-peptone solution pH 7.0. From sample prepared in this way, qualitative examination and quantitative enumeration were performed. The plate-count method was used in the study. For the determination of total aerobic microbial count (TAMC) soya bean digest agar was used and for determination of total combined yeast / moulds count (TYMC) Sabouraud – dextrose agar was used. The volume of 0.1 mL of the sample prepared was spread on the surface of the media, at least 2 Petri dishes for each media for each dilution of sample. After incubation (for bacteria – five days in temperature 30–35°C; for yeast / moulds – five to seven days in temperature 20–25°C), the arithmetic mean of the counts per medium and the number of CFU (colony forming unit) in original inoculum was calculated. For qualitative examination, 10 mL of prepared sample was added to 100 mL of casein soya bean digest broth. The further procedure was dependent on the determination of the absence or limited occurrence of specified microorganism that may be detected:

S. aureus – incubation (24 h, 35°C) and transfer on Mannitol Salt Agar;

P. aeruginosa – incubation (24 h, 35°C) and transfer on Cetrimide Agar;

E. coli – incubation (24 h, 35°C) and transfer to MacConkey broth (incubation 48 h , 44°C), then transfer on MacConkey agar;

Salmonella spp. – incubation (24 h, 35°C) and transfer to Rappaport Vassiliadis broth (incubation 24 h, 35°C) then transfer on xylose, lysine, deoxycholate agar;

Enterobacteriaceae – incubation (2–5 h, 20–25°C), transfer to enterobacteria enrichment broth-Mossel (incubation 24–48 h 35°C), transfer on violet red bile glucose agar.

Further identification was carried out using automatic Vitek 2 system Compact (bioMerieux). Results were evaluated in accordance with criteria listed in Table 1.

RESULTS

A total of 1165 samples of dietary supplements of different compositions were assessed. Most of them (67.5%) were supplements containing ingredients of natural (herbal) origin. The analysis showed that 6.5% of all samples under study failed to comply with the requirements in place.

The samples were found to have exceeded the maximum acceptable microbial count. Pathogenic microorganisms were also detected (Table 2). None of the samples under study contained *S. aureus* or *Salmonella* spp.

The most common nonconformity, identified in a half of all non-compliant samples, was the exceeding of the maximum acceptable aerobic bacterial count. Excessive fungal counts were identified less frequently. A small amount of samples was found to contain excessive counts of Gram-negative *Enterobacteriaceae* bacteria. The presence of *E. coli* was confirmed in six samples (Table 2).

Table 1. Limits for microbiological contamination.

Document	Total aerobic microbial count (CFU/g)	Total yeast/moulds count (CFU/g)	Specified microorganisms
Ministry of Health Regulation (from 13. 01. 2003). Dietary products	10^4 - 10^5	10^2 - 10^3	Absence of *Salmonella* (10 g) coli group 10^a - 10^a CFU/ g *S. aureus* 10^a-10^a CFU/ g
Regulation commission UE number 1441/2007. Food	-	-	*Listeria monocytognes* 10^a CFU/ g
Criteria according to the customer	10^4	10^2	Absence of *Salmonella* (10 g) Absence of *E. coli* (1 g) Absence of *S. aureus* (1 g) Not more than 10^a *Enterobacteriaceae* and certain other Gram-negative bacteria (1 g)

Among 381 taken under examination samples not containing natural ingredients, microbiological contamination was detected in only two products . The cause of contamination was exceeding maximum levels of mould counts.

DISCUSSION

Dietary supplements are foodstuffs and must therefore conform to the definition of food contained in Regulation (EC) No. 178/2002 of the European Parliament and of the Council of 28 January 2002, which provides that: "food" (or "foodstuff") means any substance or product, whether processed, partially processed or unprocessed, intended to be, or reasonably expected to be, ingested by humans"(3).

According to the definition contained in the Act of 25 August 2006, dietary supplements are marketed in dosage forms including capsules, tablets, coated tablets and similar forms such as powder-containing sachets, ampoules with liquid, drop bottles and other forms intended for consumption in small measured amounts (4). As the above shows, dietary supplements are available in forms typical for pharmaceutical products, i.e., items which are required to satisfy microbiological purity criteria defined in the Polish Pharmacopoea. Under EU laws, each Member State decides on its own whether a given substance is to be classified as a medicinal product or a dietary supplement. Supplements are intended specifically to make up for nutritional deficiencies or to maintain adequate levels of nutrients in the diet. They are foodstuffs which are associated by patients with caring for their health. Since dietary supplements are routinely purchased in pharmacies, where they stand on the shelf next to medicinal products, they should be as scrupulously controlled in terms of microbiological contamination as medicinal products.

Dietary supplements are purchased as well as by healthy people and those who are ill, or suffering from compromised immunity. The presence of pathogenic microorganisms in such products may thus be dangerous and potentially lead to infections. Another point to consider is the fact that there are supplements formulated specifically for children and infants. Microbiological contamination can also reduce the quality and stability of the finished product.

According to a report published by the European Commission from 2008, the number of substances used in the production of dietary supplements equals ca. 400. In terms of composition, they can be classified into several groups: vitamins and minerals, amino acids, enzymes, pro- and prebiotics, unsaturated fatty acids and supplements containing herbal ingredients (5). In the studies reported here, dietary supplements based on herbal ingredients constituted 67.5% of study samples. They were formulated with a variety of ingredients including European blueberry fruit, hawthorn fruit, raspberry fruit, ginkgo biloba, Jerusalem artichoke tubers and cranberry fruit. Some of these ingredients are registered as stand-alone herbal medicinal products and therefore, they must comply with microbiological requirements set out in the Polish Pharmacopoeia.

Annex I to the Commission Regulation (EC) No. 1441/2007 of 5 December 2007 specifies microbiological criteria for different groups of foodstuffs, however without identifying a separate group of dietary supplements. These can only be classified as belonging to one of the groups: 1.3. Ready-to-eat foods unable to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (6). Furthermore, Annex 7 to the Regulation issued by the Minister of Health, Republic of Poland on 13 January 2003, establishes maximum acceptable limits of microbiological contamination for foodstuffs, however without address-

Table 2. Microbiological contamination of food supplement.

Number of samples of incompatibility (n = 1165)	Frequency of the causes of nonconformities (n = 76)	Causes of incompatibility
38 (3.3%)	50.0%	Exceeded number of bacteria
19 (1.6%)	25.0%	Exceeded number of fungi
6 (0.5%)	8.0%	Presence of *E. coli*
13 (1.1%)	17.0%	Exceeded number of bacteria species of *Enterobacteriaceae*
76 (6.5%)	100%	Total

ing dietary supplements directly. These can only be classified as dietary products for which contaminant limits are provided (7) (Table 2). Consequently, manufacturing plants may – but are not formally required to – test dietary supplements to determine their microbiological contamination levels. As it is, companies may also adopt their own criteria for microbiological contamination and for the presence of pathogenic bacteria. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 sets maximum levels for certain contaminants in foodstuffs (e.g., nitrates, metals, dioxins, mycotoxins) (1).

Mycotoxins are produced by some species of mould (including genera: *Aspergillus, Penicillium* and *Fusarium*), which can be contamination of dietary supplements. The toxins can be carcinogenic and mutagenic substances. They can also cause acute and chronic poisoning, allergies, diseases of the respiratory and digestive systems, and liver damage (8, 9). As demonstrated by studies, out of 1165 tested dietary supplements, maximum acceptable limits for mould were exceeded in 19 cases. Tournas and et al. (10) reported that 78% of the ginseng herb supplements, 100% of the Siberian, 56% of the Chinese and 48% of the American ginseng root samples showed fungal contamination. Fungi found in the ginseng herb were *Alternaria alternate*, *Aspergillus niger*, *Aspergillus* spp., *Cladosporium* spp., *Penicillium* spp., *Rhizopus* spp. and yeasts (10). In addition, in another study, it was indicated that 60% of samples of milk thistle dietary supplements were contaminated with fungi (11).

As laid down in Commission Regulation (EC) No. 1441/2007, foodstuffs should not contain microorganisms, their toxins or metabolites in amounts that pose unacceptably high risks to human health. Unfortunately, however, maximum acceptable limits are not specified (6). Tests showed 13 samples to contain Gram-negative *Enterobacteriaceae* bacteria, while 6 samples were contaminated with *E. coli*. The samples in which Gram-negative bacterial contamination limits were exceeded and *E. coli* was detected were dietary supplements containing herbal ingredients (Jerusalem artichoke tubers and European blueberries). The contaminants may have their source in the natural environment (water, soil). Crops may also become indirectly contaminated through poorly composted organic fertilizers. Ruminant feces may be a source of contamination with *E. coli* bacteria which form a part of their natural intestinal flora.

The Chief Sanitary Inspector keeps a register of products covered by the notification of the first market placement (including dietary supplements) at the territory of Republic of Poland. Data from this registry show an increase in the number of registered dietary supplements. In the period 2007–2012 the number was respectively 791, 773, 1906, 1260, 983, 1444 (12). The products are used not only by healthy individuals but also patients with immune deficiencies and children. As the tests showed, the percentage of microbiologically contaminated samples of dietary supplements was 6.5%. Patients taking dietary supplements should be able to feel confident that they ingest products that are safe and pose no risk to their health.

The obligation to ensure the safety of dietary supplements should rest with the manufacturer, as such products should not pose a health risk to consumers. The dietary supplements are produced by various manufacturing sites: pharmaceutical companies using GMP systems in their manufacturing facilities but also by food industry cooperatives. An appropriate legal framework should, however, be adopted to precisely define maximum acceptable microbiological contamination limits in dietary supplements.

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USE OF MEDICINES AMONG STUDENTS OF HIGH SCHOOLS IN THE CZECH REPUBLIC

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Abstract: The aim of the study was to examine the prevalence and type of medicines used by high school students in the Czech Republic and to analyze association between use of medicines during last 6 months and self medication with different socio-demographic and lifestyle aspects. A cross-sectional study was conducted in high schools from two regions and three towns in the Czech Republic. The study sample was a student population of nine selected high schools. The study was approved by the local Ethics Committees and school board and was conducted in compliance with the ethical principles of the Declaration of Helsinki. Questionnaires respecting anonymity were distributed to the students during the regular class period. Students answered closed and open questions divided into six sections focussing on their experience with chronic and irregular use of medicines, medicines use during last six months, conditions treated, type and source of used medicines. A modified log-linear Poisson regression model with a robust variance estimator (sandwich) was used for statistical analysis. In total, 979 students, aged 14–21 years (mean age 17 years) participated in the study: 32% were male and 68% female; 19% of students reported regular use of medicines for chronic disease. The use of medicines during the last 6 months was reported by 83% of students; 42% of them reported the use of both prescription and over the counter (OTC) medicines; 22% the use only of OTC, and 31% only prescription medicines. Allergy, asthma, disorders of the thyroid gland, atopic dermatitis, and hypertensive diseases were among the most frequent long-term diseases. The most frequent groups of medicines used for the treatment of long-term diseases were systemic antihistamines, thyroid preparations, and respiratory drugs. Nonsteroidal anti-inflammatory medicines, ibuprofen and paracetamol were the most frequently used medicines during last six months. The study results showed quite extensive use of both prescription and OTC medicines in students of high schools between 14-21 years of age in the Czech Republic. Statistically significant associations were found between use of medicines during last 6 months and type of school, sex and chronic diseases, and between self-medication and sex.

Keywords: high school students; medicine-taking behavior; non-prescription drugs; nonsteroidal anti-inflammatory drugs

In the 1950s and 1960s consumption of medications was influenced by doctors and health care centres. Patients were used to receiving advice and prescriptions from doctors and took a passive role with respect to selection of medication and management of their disease. During the last 40 years the situation has changed dramatically not only due to economic reasons but also due to a change of attitude of patients, who are willing to take responsibility in health related matters. Since the 1990s, a growing number of products have been granted non-prescription status and self medication has become a standard part of life (1-3).

Most studies focussed on adolescents medicine-taking behavior that have been carried out in the last two decades, in Western European and Arabian countries, suggest that a significant increase in the use of prescription and use and abuse of non-prescription medicines has emerged among adolescents (4-15). Limited data is available from Central and Eastern European countries.

The aim of this study was to examine the prevalence of medication use and medicine taking behavior in students of high schools in the Czech Republic, compare the data with the data from similar studies conducted in other countries and to find out if socio-demographic characteristics of subjects and lifestyles have any influence on medicines use.

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METHODS

A cross-sectional study was conducted in nine high schools from two regions and three towns in the Czech Republic (CZ).

Participants

The planned number of high schools students to be included in the research was about one thousand. High school in CZ is defined as a school with 4 years of education which ends with a graduation examination (equivalent to A-level examination in the United Kingdom). To enter high school, students have to complete 9 years of elementary school and pass entry exam to high school. Usual student ages range from 14-19 years. In order to cover standard stratification of high schools in the district towns in CZ, three different types of secondary educational schools were included in the study: general high school (called a gymnasium in CZ), nursing school and avocational high school. As the average number of students in a classroom in secondary schools in CZ is about 30, at least 36 classes (1^{st}-4^{th}) were to be involved to meet expectation of the planned 1000 students. Based on this calculation, nine schools, from three different district towns (Hradec Kralove, Opava, and Krnov) in two different regions (Kralovehradecky, Moravskoslezsky) were selected for the research. The researchers selected towns where they could conduct the research in person in the schools. Selected schools were invited to participate by personal visit of researchers to the schools. After mutual oral informal consent, formal written consent from the chairman of the school board was obtained to conduct the study. The dates and time were agreed in advance with the class teachers. In each of the school one classroom of each of grade (1^{st}-4^{th}) was randomly chosen for research. The research was conducted during a regular class period and all students that were present at school were invited to participate in the research. The study was approved in advance by the local Ethics Committees. The students were informed in advance by the class teacher about the planned study, that the data were to be collected during a regular class period and that participation in the research was voluntary. None of students who were present on that day at school refused to attend the study. At the beginning of the data collection, the researcher made a brief presentation to the students related to the aim of the study, how the data would be analyzed and used in the future. It was also emphasized that the survey was anonymous and for the purpose of the study it was desirable to collect real life data thus students were encouraged to enter true responses or not to respond at all, or in case of doubt or an unclear question to ask the researcher for further clarification. Then, the anonymous questionnaire was distributed to the students to be completed. At all times, one of the researchers and a class teacher were present in the classroom. After collection of completed questionnaires, the researcher distributed to the students a prepared brochure with basic information focussing on the risks and benefits of using medicines, adverse drug reactions, guidance for proper use of medicines and information about where students can find additional useful information regarding medicines use. Then, the researcher gave short presentation on this topic followed by a discussion with students.

Study questionnaire and validation procedure

A structured questionnaire was developed through a series of focus group discussions with subject experts and following a review of literature in PubMed and Web of Science [4-15]. The questionnaire was content-validated by the subject experts and after pilot testing with 30 questionnaires some questions were excluded or modified to enable better understanding by the students. The questionnaire had multiple choice and open questions, 48 questions for male students and 51 questions for female students and was divided in the following sections:

1. Socio-demographic details;
2. Lifestyle habits;
3. Prevalence of chronic diseases and chronic medication;
4. Use of prescription and non-prescription medication in last 6 months and health conditions treated;
5. Source of medication procurement and medication recommendation;
6. Prevalence and frequency of OTC use.

A list of options was included for sections 3 to 4, following an initial review of literature, which indicated the commonly reported issues among self-medicating students. The listed drugs were further classified according to anatomical-therapeutic-chemical (ATC) classification in the results.

Statistical analyses

Descriptive statistics were used to summarize the data collected from the questionnaire. To estimate prevalence ratios a modified log-linear Poisson regression model with a robust variance estimator (sandwich) was used (16). The package Sandwich (17) implemented in the R system for statistical

computing was used for the data analysis (18). Type of school, age, sex, smoking habits, time spent on sport, TV and internet, use of illicit drugs, mother's and father's education were included in the model as main socio-demographic and lifestyle characteristics of subjects and their influence on medicines use and self medication use has been assessed. Several questions had multiple responses and therefore the percentages did not add up to a total of 100%. Since the participants did not respond to every statement in the questionnaire, there were differences in the value of n (the total population of respondents) for individual questions.

RESULTS

In total, 979 students, aged 14–21 years (mean age 17 years) from 9 schools participated in the study.

Out of 979 students 32% were males and 68% females. Not all students responded to all of the questions thus demographic data and further statistical analysis included only the full answers to each of the questions. The main socio-demographic characteristics are summarized in Table 1.

Out of 964 students who responded, 177 (19%) reported regular use of medicines for chronic disease. Allergy, asthma, disorders of the thyroid gland, and atopic dermatitis were among the most frequent long-term diseases reported. The groups of medicines most frequently used for the treatment of long-term diseases were systemic antihistamines, thyroid preparations, and respiratory medicines (Table 2).

The use of medicines during last 6 months was reported by 796 (83%) of the students, 342 (43%) of whom reported the use of both prescription and OTC medicines, 249 (31%) only the use of OTC

Table 1. Demographic characteristics.

Age	***n (mean)***
14 - 21 years	979 (17)
Type of school	***n (%)***
High school	470 (48.0)
Nursing school	302 (30.9)
Vocational school	207 (21.1)
Sex	***n (%)***
Female	669 (68.3)
Male	310 (31.7)
Do you suffer any chronic disease?	***n (%)***
Yes	188 (19.3)
No	787 (80.7)
Do you use regularly medicines for chronic disease?	***n (%)***
Yes	177 (19.4)
No	737 (80.6)

Table 2. Prevalence of chronic disease and regular use of medicines.

Prevalence of long-term disease	***Total n (%)***
Allergy	79 (8.1)
Asthma	26 (2.6)
Disorders of the thyroid gland	20 (2.1)
Atopic dermatitis	8 (0.8)
Others (less than 7)	55 (5.7)
Students without chronic disease	787 (80.7)
Total	***975 (100)***
Most frequent groups of medicines used for treatment of chronic diseases	***Total n (%)***
Antihistamines for systemic use/Anti-allergic drugs	83 (8.5)
Thyroid preparations	16 (1.6)
Respiratory/Anti-asthmatics	14 (1.4)
Mineral supplements 7 (0.7)	
Others (less than 7)	57 (5.8)
Students without chronic medication	787 (80.7)
Total	***964 (100)***

Table 3. Health conditions for using medicines by students during last 6 months*.

Health conditions	Number (%)
Headache	406 (41.5)
Cold and sore throat	373 (38.1)
Dysmenorrhoea	276 (28.2)
Fever	248 (25.3)
Vomiting/Diarrhoea	81 (8.3)
Migraine	79 (8.1)
Toothache	53 (5.4)
Muscle/Joint aches	50 (5.1)
Stomach aches	35 (3.6)
Contraception	3 (3.4)
Others	115 (11.7)

* Students indicated all health problems they suffered during last 6 months. In total 796 students responded.

medicines, and 205 (26%) took only prescription medicines. For subsequent question related health conditions for which students used medication during last 6 months, the students could enter all health conditions and medicine used. Thus, the data collected for this section represented prevalence of each of health condition during last 6 months and medicines taken to treat this condition. Among the most frequent health conditions for using medicines during last 6 months reported by students were headache 406 (41.5%), cold and sore throat 373 (38.1%), dysmenorrhoea 276 (28.2%), fever 248 (25.3%), vomiting/diarrhoea 81 (8.3%), migraine 79 (8.1%), toothache 53 (5.4%), muscle/joint aches 50 (5.1%), stomach aches 35 (3.6%), contraception 33 (3.4%) and the others 115 (11.7%) (Table 3).

Out of 796 students 685 (86%) remembered the exact names of the medicines they used. Where the students indicated a brand name of a medicine, the generic names were used for statistical purposes. Nonsteroidal anti-inflammatory medicines, ibuprofen and paracetamol, were the most frequently used medicines in general (Table 4).

About 10% of students (99) noted that they never used OTC medicines, 3% (29) used OTC medicines every day and a high majority of students use OTC medicines occasionally or sometimes. Absolute results and results per different categories (sex, type of school and students with and without chronic disease) are summarized in Table 5.

Out of 979 students, 737 (75.3%) admitted self-administration of medicines during their lifetime. The most frequent medicines that students used were ibuprofen (n = 470) and paracetamol (n = 263). The three most common reasons for self medication reported were immediate help and relief (n = 381), the simplest solution in that situation (n = 189), and familiarity with the safe use of medicines (n = 140). The student's mother was the most frequent person who recommended and dispensed the medicines to students (n = 412), followed be physician (n = 275), and pharmacist (n = 173). The main source of medication was the home pharmacy cabinet (n = 681) and pharmacy (n = 162). Of the female students, 446 admitted use of medicines for menstrual discomfort, with ibuprofen (n = 389), paracetamol (n = 53), and metamizole (n = 47) the most frequently chosen.

The data showed statistically significant association between the medicines used during last six months and type of school, sex and chronic diseases. Students who attended nursing schools tended to use fewer medicines than the students from other types of schools; females and students with chronic disease used more medicines. Students from nursing schools and students with chronic disease used less OTC medicines than other students during last six months. As regards students' self-medication during their lifetime, a statistically significant association was found for female students (Table 6).

DISCUSSION

Statistical analyses

The most common method of modelling binomial health data in cross-sectional studies today is logistical analysis, which works very well if one wants to model the ratio of odds instead of the ratio of probabilities. It also yields a good approximate analysis if one is interested in the ratio of probabilities of a rare event. However, if the event is not rare, and one is interested in the ratio of probabilities (i.e., prevalence ratio), then the logistic approximation will be poor because the odds ratio is a poor estimator of the probability ratio. Several authors recommended using the log-binomial model (i.e., binomial model with log link function), which directly models the prevalence ratio. However, this model is not widely used as it often fails to converge. Instead, Poisson regression may be a different natural choice for fitting a log-linear model, since it estimates prevalence ratio and since most medical applications of the Poisson distribution arise *via* the Poisson approximation to the binomial distribution (19). The estimating equations for Poisson regression are unbiased when the response variable is binary rather than

Table 4. Medicines taken during last 6 months.

Health condition	Generic name	Number	ATC code
Headache	Ibuprofen	321	M01AE01
(n = 406)	Paracetamol	100	N02BE01
	Naproxen	5	M01AE02
	Nimesulide	5	M01AX17
Cold and sore throat	Dichlorobenzyl alcohol + Amylmetacresol	68	R02AA20
(n = 373)	Benzalkonium chloride + Menthol + Essential oils	40	R02AA20
	Antibiotics	35	J01
	Benzydamine	30	A01AD02
	Butamirate + Guaifenesin	28	R05FB02
	Benzoxonium chloride + Lidocaine hydrochloride	26	R02AA20
	Paracetamol	23	N02BE01
	Paracetamol + Phenylephrine	22	N02BE51
Fever	Paracetamol	186	N02BE01
(n = 248)	Ibuprofen	51	M01AE01
	Aspirin	14	N02BA01
	Antibiotics	9	J01
Dysmenorhoea	Ibuprofen	214	M01AE01
(n = 276)	Paracetamol	26	N02BE01
	Metamizol + Pitofenone	25	A03DA02
	Nimesulide	8	M01AX17
Vomiting/ Diarrhoea	Carbo medicinalis	29	A07BA5
(n = 81)	Other intestinal adsorbents	27	A07BC05
	Intestinal anti-infectives	22	A07AX
	Antidiarrheal microorganisms	4	A07FA01
	Loperamide	3	A07DA03
Migraine	Ibuprofen	55	M01AE01
(n = 79)	Paracetamol	9	N02BE01
	Metamizole	4	N02BB02
	Sumatriptan	3	N02CC01
	Nimesulide	3	M01AX17
Muscle/Joint ache	Ibuprofen	19	M01AE01
(n = 50)	Magnesium sulfate	5	A12CC30
	Ketoprofen	4	M01AE03
	Paracetamol	3	N02BE01
Toothache	Ibuprofen	45	M01AE01
(n = 53)	Paracetamol	3	N02BE01
	Nimesulide	2	M01AX17
	Paracetamol + Propyfenazon	2	N02BE51
Stomach ache	Other intestinal adsorbents	7	A07BC05
(n = 35)	Ibuprofen	5	M01AE01
	Calcium carbonate	4	A02AD01
	Metamizole	4	N02BB02
Others	Contraceptives	33	G03
(n = 148)	Cetirizine	21	R06AE07
	Antibiotics	16	J01
	Loratadine	7	R06AX13
	Desloratadine	6	R06AX27
	Levocetirizine	5	R06AE09

Poisson, and thus lead to consistent estimation of the prevalence ratio. However, when used to estimate prevalence ratio from binary data, Poisson regression gives standard errors that are too large, because the variance of a Poisson random variable is always larger than that of a binary variable with the same mean. Thus this bias was minimized by using the model-robust sandwich estimator used by Zeileis (17).

Bias of the study

The authors are aware of several limitations of this study. Survey questions on medicines use do have methodological limitations affecting their validity as health status indicators, specifically recall period and motivation of the respondents to provide correct answers. To try to reduce bias some cross-check questions were included in the ques-

Table 5. Frequency of use of OTC medicines (%).

Frequency of use of OTC medicines	Number of students	Female	Male	High school general	School for nurses	Other schools	Students with chronic disease	Students without chronic disease
	(n = 958)	(n = 652)	(n = 299)	(n = 456)	(n = 298)	(n = 199)	(n = 180)	(n = 768)
Never	99 (10.3)	46 (7.1)	53 (17.7)	51 (11.2)	28 (9.4)	20 (10.0)	12 (6.7)	87 (11.3)
Occasionally (less than 1× a month)	574 (59.9)	428 (65.6)	144 (48.2)	269 (59.0)	187 (62.7)	118 (59.3)	93 (51.7)	477 (62.2)
Sometimes (1× a month)	112 (11.7)	90 (13.8)	22 (7.4)	42 (9.2)	41 (13.8)	29 (14.6)	35 (19.4)	77 (10.0)
Often (1× a week)	22 (2.4)	15 (2.3)	3 (1.0)	7 (1.5)	6 (2.0)	5 (2.5)	7 (3.9)	11 (1.4)
Every day	29 (3.0)	15 (2.3)	13 (4.3)	17 (3.7)	6 (2.0)	5 (2.5)	11 (6.1)	16 (2.1)
Others	122 (12.7)	58 (8.9)	64 (21.4)	70 (15.4)	30 (10.1)	22 (11.1)	22 (12.2)	100 (13.0)

Table 6. Influence of socio-demographic characteristics of subjects and lifestyles on the use of medicines.

All medicines use during last 6 months			
Terms	Level	p-value	PR (95% Cl)
School	0 – school for nurses		
	1 – other schools	0.014	1.09 (1.02-1.17)
Sex	0 – female 1 – male	< 0.001 0.85	(0.79-0.92)
Chronic disease	0 – yes, 1 – no	< 0.001 0.84	(0.80-0.88)
OTC use during last 6 months			
Terms	Level	p-value	PR (95% Cl)
School	0 – school for nurses		
	1 – other schools	< 0.001	1.34 (1.18-1.52)
Chronic disease	0 – yes, 1 - no	0.002	1.23 (1.08-1.41)
Students self-medication in their lifetime			
Terms	Level	p-value	PR (95% Cl)
Sex	0 – female 1 – male	0.023	0.92 (0.86-0.99)

* To estimate prevalence ratios a modified log-linear Poisson regression model with a robust variance estimator (sandwich) was used. Type of school, age, sex, smoking habits, time spent on sport, TV and internet, use of illicit drugs, mother's and father's education were included in the model as main socio-demographic and lifestyle characteristics of subjects (in Table as terms). Statistically non significant terms not included in the Table.

tionnaire to show consistency of the responses. The results of some questions were also compared with general population statistics (e.g., prevalence of some chronic diseases). It is acknowledged that the data collected from 3 types of schools, 979 students, 3 different districts and 2 different regions might not be a sufficient sample to represent the whole population of Czech high school students. In our study 68% of female students participated in the study and this number was caused by selection of the schools (a majority of students in nursing schools are female students). However, further research in the field is needed to get more proportional data related to both genders. Questionnaires were completed under presence of researchers and a teacher, which might imply that the students provided social desirable answers (social acquiescence). To minimize this bias, neither teacher nor researcher have been checking the answers during the survey and the questionnaires were collected by students and provided directly to the researcher. Students were also encouraged to complete true data or not to respond rather than complete desirable or fabricated data. Only 33 female students reported use of contraceptive pills but quite high use of medication for menstrual discomfort. The seemingly low number of contraceptive users might be caused by an information bias, as use of contraception might not have been perceived as chronic treatment.

Comparison with other studies

The data from some other studies including children, high school and university students proved that a large proportion of students are exposed to medicines, often since childhood, and self administer the medicines without parents' supervision (15, 20–23). Comparable with other studies the main conditions for medicines use were headache, cold and sore throat and pain (7, 21). In concordance with other studies carried out in Sweden, England, Brazil, Denmark, and UAE, analgesics and antipyretics (ibuprofen and paracetamol) were most commonly used non-prescription medicines (7, 10, 15, 21, 27). The data regarding prevalence of allergy and asthma is comparable with world statistics related to these diseases (24, 25).

Although other studies have used different recall periods, which limits comparison, the prevalence of self-medication in our sample is comparable to that reported in Canada (58.3 to 75.9%), lower than Maltese (90.3%) and UAE (89.2%) students and higher than that observed in German (57%) or Brazilian students (55.8%; 56.6%) (13–15, 26–28). We have not found an increase in self-medication with age as reported by authors among Danish and Kuwait students (10, 29) but we have found significant difference between gender (female) similar to other literature sources (7, 14, 30).

As regards the frequency of OTC use, our data were similar to those reported by Swedish authors (7). The home medicine cabinet was the most common source of medicines and the mother was the most frequent person who dispensed medicines to these populations in general (7, 12, 15, 26, 31). Similar to some other authors, we have not found any relation between use of OTC medicines in students and their lifestyle habits or socio-economic differences or between use of medicine and age and between the use of OTC medicines and by the presence of chronic illnesses (30–33). No literature data were found regarding comparison of the data from different types of high schools.

CONCLUSION

This is the first research study to explore the self-medication practices among students in the Czech Republic and provides baseline data critical in creating awareness about the risks and benefits of self-medication. The prevalence of self-medication among the high school students in CZ is high but comparable with the data presented by other countries. The use of medicines in students in CZ was not associated with personal and lifestyle characteristics and age, but was associated with sex, chronic disease and the type of school the students attended. Additional studies are needed to include all types of schools and proportionate gender participation. It is extremely important that students are aware of the risk and benefits of medicines as well as of proper use of medicines and self-diagnosis to avoid potential abuse, misuse or improper use of medicines. Health care providers, parents, pharmacists and teachers in CZ should be actively involved in early health education strategies for establishing responsible use of medicines in this age group.

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Conflict of interest None declared.

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SHORT COMMUNICATION

CYTOTOXICITY AND ANTIGLUCOSIDASE POTENTIAL OF SIX SELECTED EDIBLE AND MEDICINAL FERNS

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Globally, bioactive phytochemicals are gaining popularity among consumers, as evidenced by the steady rise in the demand for botanical dietary supplements. One driving force behind this is the perception of herbal medicine being a safe and relatively inexpensive alternative in the management of human diseases (1, 2). There is currently intensive effort worldwide to search for bioactive phytochemicals for potential applications in the development of novel or alternative nutraceutical, cosmetic and pharmaceutical products (3, 4). Phytochemicals with cytotoxic and antiglucosidase properties, for example, can be exploited in the formulation of chemopreventive and antidiabetic drugs (5, 6) as well as in the development of nutraceuticals and functional food (7-9).

Ferns are rich sources of natural products with diverse bioactivities, including anticancer, antibacterial, antioxidant, and antiinflammatory activities (7). Nevertheless, less emphasis has been given to ferns than to other plant groups in bioprospecting research aiming at discovering natural therapeutic or bioactive agents. Many ferns have been traditionally used as remedies for human diseases as well as being consumed as vegetables. Thus, ferns are potential candidates for the discovery of bioactive constituents and bioactivity which can be exploited for the development of nutraceutical, cosmetic and pharmaceutical products (7, 8).

This study focused on six medicinal and edible ferns, namely *Christella arida*, *Christella dentata*, *Cyclosorus interruptus*, *Microsorum punctatum*, *Nephrolepis acutifolia* and *Pleocnemia irregularis*. The leaf and root of *C. arida* are traditionally used to treat dysentery and skin diseases (10). *C. dentata* is an edible fern (11), which is also a folk remedy for skin diseases (12). *C. interruptus* is a medicinal plant used for treating sores, burns, liver diseases, gonorrhea, cough, and malaria (13). *C. interruptus*-derived coumarin derivatives are cytotoxic to human nasopharyngeal carcinoma (KB) cell line and exhibited antibacterial activity (14). Juice extracted from the fronds of *M. punctatum* is used as purgative, diuretic, and wound healing agents (15). *N. acutifolia* and *P. irregularis* are both edible ferns (16, 17). *P. irregularis* is also used to treat diarrhea, skin diseases (18) and weak muscles (19). At present, the anticancer potential of these six ferns, except *C. interruptus*, has not been reported. Neither has the antiglucosidase activity of any of the six ferns been investigated. Information on the polyphenol, hydroxycinnamic acid, flavonoid, and proanthocyanidin contents of the six selected ferns is scarce. Such phytoconstituent classes are known to have important health-promoting and therapeutic effects (7, 9, 20). Hence, our goals were to fill in current gaps of knowledge about the bioactivities of these ferns, in addition to identifying a promising fern species which can be used in the future investigations for the isolation of active compounds. The specific objective of this study was two-fold: first, to assess the cytotoxicity and antiglucosidase activity of the aqueous extracts of the six selected ferns; second, to determine whether such bioactivities can be

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attributed to the contents of phenolics, hydroxycinnamic acids, flavonoids, and proanthocyanidins in the extracts.

MATERIALS AND METHODS

Plant samples

Healthy specimens of six ferns, namely *Christella arida* (D. Don) Holtt (Thelypteridaceae), *Christella dentata* (Forsk.) Brownsey & Jermy (Thelypteridaceae), *Cyclosorus interruptus* (Willd.) H. Ito (Thelypteridaceae), *Microsorum punctatum* (L.) Copel. (Polypodiaceae), *Nephrolepis acutifolia* (Desv.) Christ. (Nephrolepidaceae), and *Pleocnemia irregularis* (C. Presl.) Holtt. (Tectariaceae) were gathered from the countryside of Bidor town, Malaysia, by T.-T. Chai and F.-C. Wong in February 2013. The species of the ferns were authenticated by H.-C. Ong.

Preparation of aqueous extracts

Fern samples were oven-dried at 45°C for 72 h and then ground to powder with a Waring blender. Aqueous extracts were prepared by suspending the fern powder in autoclaved, deionized water at a ratio of 1 : 20 (dry weight : volume). The mixture was incubated in a 90°C water bath for 1 h. The extract was then vacuum-filtered and the filtrate obtained was centrifuged at 7830 rpm at 4°C for 5 min. Next, the supernatant collected was freeze-dried to constant weight. The freeze-dried extract was dissolved in deionized water to prepare aliquots of 50 mg/mL, which were then stored at -20°C until further use.

Cytotoxicity and antiglucosidase activity

The cytotoxic activity of the fern extracts was assessed by conducting a methylthiazol tetrazolium (MTT) assay using a human chronic myelogenous leukemia cell line (K562) as previously described (21). Among the six selected ferns, the cytotoxicity of only *C. interruptus* was previously investigated (14). The cytotoxic effects of these ferns on a leukemia cell line have never been reported in the literature. More importantly, this choice of cell line was in line with our long-term research interest to identify cytotoxic compounds from ferns which are useful for the development of therapeutic agents against leukemia. 5-Fluorouracil (5FU), an anticancer drug, was used as the positive control. EC_{50} value, defined as the extract concentration required for achieving 50% cytotoxic activity, was determined by using linear regression analysis.

Glucosidase inhibitory activity was determined as described in (22). Myricetin and acarbose were used as the positive controls. The effectiveness of myricetin as an inhibitor of yeast and mammalian α-glucosidases has been established (23). Acarbose is an oral antihyperglycemic drug with antiglucosidase activity (24). EC_{50} value, defined as the extract concentration required for achieving 50% antiglucosidase activity, was computed by using linear regression analysis.

Phytochemical contents

Total phenolic (TP) content of the fern extracts was determined by using a Folin-Ciocalteu colorimetric assay (25). TP content was expressed as mg gallic acid equivalents (GAE)/g extract, calculated from a standard curve prepared with 0-100 µg/mL gallic acid. Total hydroxycinnamic acid (THC) content was determined by using the Arnow's reagent (26). THC content was expressed as mg caffeic acid equivalents (CAE)/g extract, computed from a standard curve prepared with 0-0.2 mg/mL caffeic acid. Total flavonoid (TF) content was determined by using an aluminum chloride colorimetric assay (27). TF content was expressed as mg quercetin equivalents (QE)/g extract, calculated from a standard curve prepared with 0-500 µg/mL of quercetin. Total proanthocyanidin (TPro) content was assessed by the acid-butanol assay (28). TPro content was calculated with the assumption that effective $E^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460 (28) and expressed as mg leucocyanidin equivalents (LE)/g extract.

Data analysis

All experiments were carried out in triplicates. Statistical analyses were performed using SAS (Version 9.2). Data were analyzed by one-way ANOVA test and means of significant differences were separated using Fisher's Least Significant Difference (LSD) test or Student's *t* test at $\alpha = 0.05$. Linear regression and correlation analyses were carried out by using Microsoft Office Excel 2007.

RESULTS

All fern extracts investigated in this study were cytotoxic toward K562 cells. Statistical analysis revealed that the EC_{50} values for cytotoxic activity of *C. dentata*, *N. acutifolia*, and *P. irregularis* extracts were not significantly different ($p > 0.05$) from that of 5-fluorouracil (Table 1). *C. arida* was the fern species with the highest EC_{50} value, which was 2.2-fold greater compared with 5-fluorouracil. Concentration-dependent increase in antiglucosidase activity was observed in all fern extracts, except *C. interruptus*, at 200-1000 mg/mL (data not

shown). The EC_{50} values for antiglucosidase activity among the fern extracts, ranked in ascending order, were: *C. dentata* < *P. irregularis* < *N. acutifolia* < *C. arida* < *M. punctatum* (Table 1). The EC_{50} values of *C. dentata* and *M. punctatum* extracts were 1.6- and 25-fold higher than that of myricetin, respectively. *C. interruptus* exhibited α-glucosidase stimulatory activity; hence its EC_{50} value was not determined. The EC_{50} value for acarbose was 2750 µg/mL (data not shown), which was markedly higher than those of myricetin and the fern extracts examined.

Phytochemical analysis found *P. irregularis* extract to be the richest in THC, TF and TPro contents (Table 2). Notably, TF, expressed as quercetin equivalents, accounted for about 37% of *P. irregularis* extract by weight. By contrast, *M. punctatum* extract had the lowest abundance of TP, THC and TF among the six extracts studied. TPro was not detectable in *M. punctatum* extract. *N. acutifolia* extract had the highest TP content, which was about 3.3-fold higher than that of *C. interruptus* and *M. punctatum* extracts. *C. interruptus* extract had the lowest, detectable TPro content, which was 56-fold lower compared with *P. irregularis*.

Only TP contents of the ferns significantly correlated with EC_{50} values for antiglucosidase activity ($R^2 = 0.82$, $p < 0.05$). No statistically significant correlations were detected between phytochemical contents of the fern extracts and EC_{50} values for cytotoxic activity.

DISCUSSION

This study demonstrated for the first time the cytotoxicity of *C. arida*, *C. dentata*, *N. acutifolia*, *M.*

Table 1. EC_{50} values of the cytotoxic and antiglucosidase activities of fern extracts, compared with 5-fluorouracil and myricetin, respectively.

Species	EC_{50} values	
	Cytotoxic activity (µg/mL)	Antiglucosidase activity (µg/mL)
C. arida	478.62 ± 39.11*	559.87 ± 21.04*
C. dentata	194.50 ± 14.74	87.48 ± 7.45*
C. interruptus	314.52 ± 6.34*	nd
M. punctatum	399.68 ± 48.60*	1345.73 ± 129.44*
N. acutifolia	190.82 ± 5.52	249.57 ± 2.67*
P. irregularis	253.85 ± 22.82	112.68 ± 1.72*
Positive control	212.86 ± 7.89 (5-Fluorouracil)	53.21 ± 0.91 (Myricetin)

Data are the mean ± standard errors (n = 3). The asterisks (*) denote values that are significantly different ($p < 0.05$) compared with the positive control, as determined by using Student's *t* test. nd = not determined.

Table 2. Phytochemical contents of fern extracts.

Species	TP (mg GAE/g)	THC (mg CAE/g)	TF (mg QE/g)	TPro (mg LE/g)
C. arida	97.21 ± 1.78^a	100.75 ± 1.28^a	324.24 ± 3.69^a	9.55 ± 0.16^a
C. dentata	107.80 ± 3.52^a	64.83 ± 1.08^b	191.52 ± 4.59^b	18.57 ± 1.79^b
C. interruptus	43.90 ± 0.62^b	9.10 ± 0.19^c	59.82 ± 1.91^c	0.44 ± 0.06^c
M. punctatum	42.57 ± 0.55^b	3.57 ± 0.04^d	15.73 ± 1.55^d	nd
N. acutifolia	143.79 ± 5.19^c	67.42 ± 0.92^b	246.67 ± 1.32^e	4.14 ± 0.25^d
P. irregularis	136.38 ± 6.65^c	119.75 ± 2.08^e	367.88 ± 2.89^f	24.71 ± 1.51^e

Data are the mean ± standard errors (n = 3). Values in the same column that are followed by different superscript letters are significantly different ($p < 0.05$), as determined by using Fisher's LSD test. TP = total phenolics; THC = total hydroxycinnamic acids; TF = total flavonoids; TPro = total proanthocyanidins;. nd = not detectable.

punctatum and *P. irregularis*. The cytotoxicity of *C. interruptus*-derived coumarins against human nasopharyngeal carcinoma (KB) cell line was previously reported (14); however, this is the first account of the fern's cytotoxicity toward K562 cell line. Different cancer cell lines respond differently even to treatment with the same cytotoxic agents (29). Hence, our study has added valuable information to current knowledge of the anticancer potential of *C. interruptus*. Hot water extracts of all six ferns analyzed in this study were cytotoxic to K562 cancer cell line. Thus, our findings suggest that these six ferns are promising sources of water-soluble and heat-stable cytotoxic agents. *C. dentata*, *N. acutifolia*, and *P. irregularis* are consumed as vegetables in Malaysia and India (16, 17, 30). Considering current interests to discover anticancer agents of food origin, future research to isolate and identify cytotoxic constituents from the three ferns will be of great value in the context of therapeutic agent development, especially for leukemia treatment.

We report for the first time the *in vitro* antiglucosidase activity of *C. arida*, *C. dentata*, *M. punctatum*, *N. acutifolia*, and *P. irregularis*. Notably, edible ferns *C. dentata*, *N. acutifolia*, and *P. irregularis* demonstrated stronger antiglucosidase activity than the other ferns. In line with current interests to search for food-derived antidiabetic natural products and management of diabetes by dietary intervention (31-34), the three edible ferns deserve more attention in future research. These ferns are not traditionally used as antidiabetic remedies. Nevertheless, owing to their α-glucosidase inhibitory activity, these ferns, when consumed, may be beneficial to the diets of diabetic patients. The water-soluble and thermally-stable nature of the α-glucosidase inhibitors in the three ferns imply that such constituents could be easily extracted with water and that their activity is likely retained after cooking with heat. In this study, we evaluated the antiglucosidase activity of the fern extracts by using yeast α-glucosidase. Yeast α-glucosidase is commercially available in pure form and has been routinely used as a model for investigating antiglucosidase potential of natural products (22, 35-37). Previous work has shown that plant extracts which inhibited the activity of yeast α-glucosidase also inhibited mammalian α-glucosidase. In addition, antiglucosidase plant extracts can significantly dampen postprandial hyperglycemia in streptozocin-induced diabetic mice (37, 38).

The presence of TP, THC, TF, and TPro in all the fern extracts, except for the absence of TPro in *M. punctatum*, implies that the cytotoxic and antiglucosidase activities of the extracts may be partly attributable to their phenolic constituents. *P. irregularis* extract, which was enriched in TF (37% by weight), showed potent cytotoxicity and concurrently exhibited relatively high antiglucosidase activity compared with the other ferns examined. Thus flavonoids may be a key group of bioactive constituents in *P. irregularis*. In this study, antiglucosidase activity of the ferns correlated with TP content, also implying that antiglucosidase activities of the ferns may be attributable to their phenolic constituents. No correlations were detected between the four phytochemical parameters measured and the cytotoxic activities of the fern extracts. It is likely that the fern extracts contained phenolic constituents that varied considerably in their efficacies or specific activity per unit mass as cytotoxic agents. Consequently, their cytotoxic activities cannot be directly predicted from their phenolic contents.

In conclusion, the cytotoxic effects of six selected edible and medicinal ferns toward K562 cell line were demonstrated for the first time. The water extracts of *C. dentata*, *N. acutifolia*, and *P. irregularis* were strong cytotoxic agents, with potency comparable to that of anticancer drug 5-fluorouracil. Antiglucosidase activity was also detected in five of these ferns for the first time. *C. dentata* had the strongest antiglucosidase activity among the ferns, which was stronger than acarbose but weaker than myricetin. This study has provided preliminary evidence that *C. dentata* is a promising source of cytotoxic and antiglucosidase agents, which warrant more in-depth investigations. To attest to their potential application as anticancer drugs, the effects of cytotoxic constituents isolated from *C. dentata* on normal cells should be confirmed. Correlation analysis suggests that antiglucosidase activity of the fern extracts was attributable to their phenolic contents. Hence, future research can consider bioassay-guided isolation of glucosidase inhibitors from phenolic extracts of the ferns, especially *C. dentata*.

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FLUORESCENCE SPECTROSCOPY STUDY OF THE INTERACTION BETWEEN OCHRATOXIN A AND HUMAN SERUM ALBUMIN IN THE PRESENCE OF FLURBIPROFEN

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Keywords: fluorescence, flurbiprofen, human serum albumin, ochratoxin A

Many natural toxins exist in the human environment, which are potentially pathogenic. One of the major groups are mycotoxins produced by some molds. This group includes ochratoxin A (OTA), produced mainly by the strain *Aspergillus ochraceus*, which commonly occurs in nature and is responsible for rotting of food articles (1, 2). OTA is responsible for the occurrence of many pathologies in the human organism. It shows a strong nephrotoxic, teratogenic, genotoxic and mutagenic effect (3, 4). As far as the chemical structure is concerned, OTA is a compound built of the amino acid α-phenylalanine connected with an isocoumarin derivative by a peptide bond (2).

Human serum albumin HSA is the main protein of human blood plasma and accounts for about 60% of the whole plasma protein. Due to the high concentration in blood serum and unspecificity of the bond, albumin plays the main role in binding and distributing endogenous and exogenous substances in the organism. The most important binding sites are sites I and II (Sudlow site I and Sudlow site II). Substances strongly bound to plasma proteins to a lesser extent penetrate into the organs (5–12).

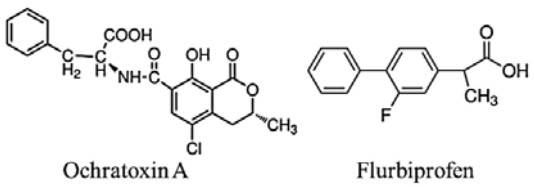

Figure 1. Structural formulas of ochratoxin A and flurbiprofen (2)

Flurbiprofen belongs to propionic acid derivatives, the most frequently represented group of non-steroidal antiinflammatory drugs. Its half-life period is about 4–5 h. The metabolism of flurbiprofen occurs mainly in the liver and its metabolites are excreted by kidneys with urine (13).

MATERIALS AND METHODS

Ochratoxin (OTA), albumin (HSA) and flurbiprofen samples were acquired from Sigma-Aldrich. Samples of OTA with a concentration of 1 μM dissolved in PBS solution were used for the analysis. PBS buffer with pH 7.4 was obtained by dissolving 8.0066 g 137 mM NaCl; 0.2236 g 3 mM KCl; 1.1357 g 8 mM Na_2HPO_4 and 0.1361 g 1 mM KH_2PO_4 in 1 liter of distilled water. Then, appropriate amounts of 0.1 M KOH or 0.1 M KCl were added to obtain the desired pH. PBS buffers were stored under cooling conditions. Before each new analysis, PBS pH was checked and the buffer was heated to 37°C. High resolution optic spectrometer of HR4000 (Ocean Optics)

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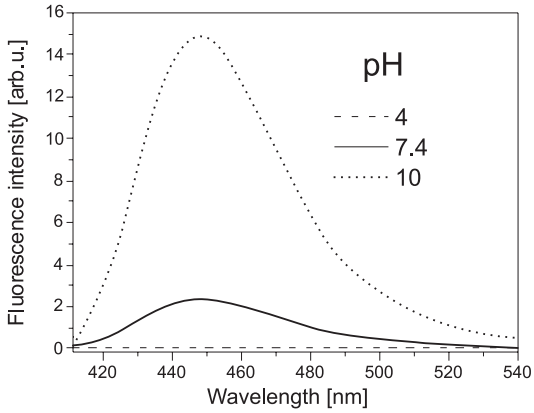

Figure 2. OTA fluorescence emission spectra in PBS at different pH. OTA concentration = 1 μM

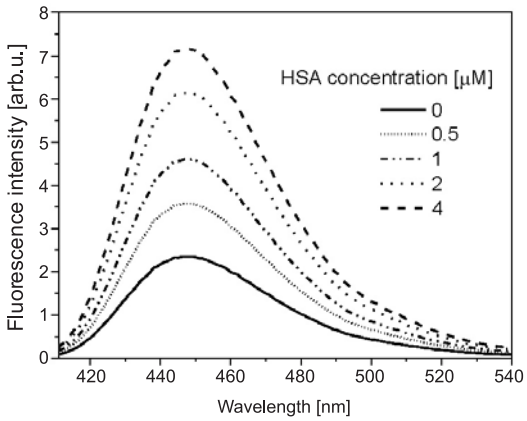

Figure 3. OTA fluorescence emission spectra in different concentration of HSA at pH 7.4

was used to register fluorescence emission spectra. Radiation with a wavelength of 395 nm was used to excite OTA (the source was a electroluminescence diode).

RESULTS AND DISCUSSION

Figure 2 presents the fluorescence emission spectra of OTA dissolved in PBS buffer with different pH values. The diagram shows that OTA in pH 4 does not fluoresce. This results from the fact that in acid pH OTA occurs only in the monoanionic form. This form of ochratoxin does not adsorb light with a wavelength of 395 nm. The intensity of OTA fluorescence increases along with the increase in pH, which is the consequence of passing from the monoanionic to dianionic form (5, 10) Quite large fluorescence can already be observed at physiological pH (7.4). This is caused by the occurrence of both monoanionic and dianionic forms at this pH. Fluorescence with a large intensity is observed at alkaline pH (pH = 10). This results from the occurrence of ochratoxin only in the dianionic form, which strongly fluoresces.

In order to examine the effect of OTA on albumin, OTA fluorescence spectra with a concentration of 1 μM were registered in the presence of albumin with different concentrations from 0 to 4 μM at physiological pH (Fig. 3). The maximum of OTA fluorescence emission spectrum in PBS buffer with a pH of 7.4 occurs at the wavelength 448 nm. Along with increasing HSA concentration, an increase in the maximum of OTA fluorescence intensity is observed. This is related to the fact that the monoan-

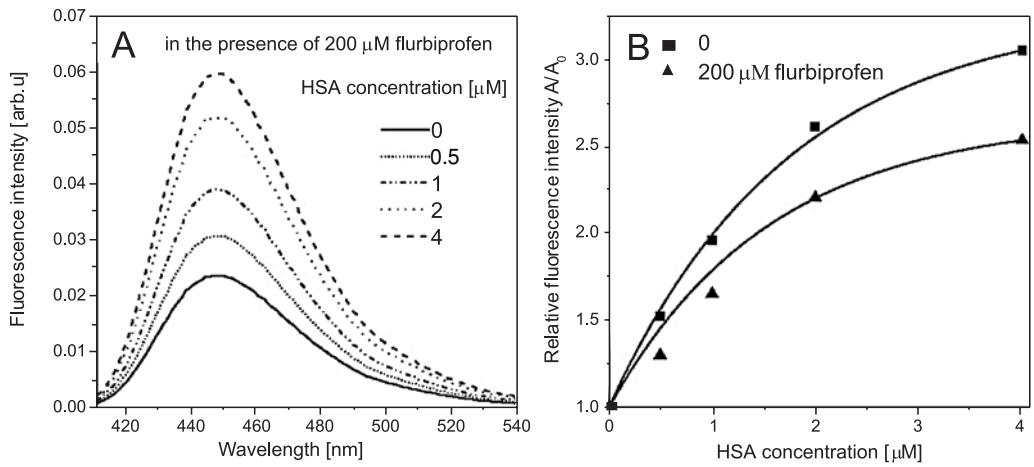

Figure 4. (A) OTA fluorescence emission spectra in HSA suspension with different concentration in the presence of flurbiprofen with a concentration of 200 μM at pH 7.4. (B) Relative increase in OTA fluorescence intensity at 448 nm depending on HSA concentration in the absence (0) and presence (200 μM) of flurbiprofen

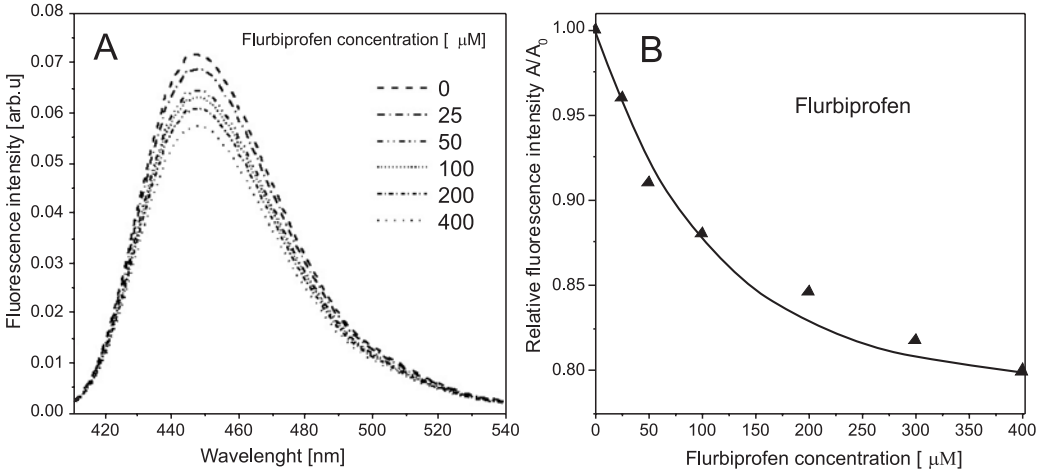

Figure 5. (A) OTA fluorescence emission spectra in HSA suspension with a concentration of 3 μM depending on the flurbiprofen concentration. (B) Relative OTA fluorescence at 448 nm in the function of flurbiprofen concentration at the constant HSA concentration (3 μM)

ionic form passes to the dianionic form by binding to protein (5, 10). Thus, fluorescence intensity depends on the amount of OTA particles bound to HSA.

Figure 4A presents the OTA fluorescence emission spectrum at pH 7.4 depending on HSA concentration after the addition of flurbiprofen with a concentration of 200 μM. Along with increasing HSA concentration, an increase in fluorescence intensity at the wavelength 448 nm is observed, but it is considerably smaller than in the case presented in Figure 3. Figure 4B presents a relative increase in OTA fluorescence intensity at the wavelength 448 nm in the function of HSA concentration, based on fluorescence emission spectra from Figures 3 and 4A. It can be concluded that flurbiprofen, which is characterized by a high affinity to the same binding sites in albumin as ochratoxin A, causes its displacement from protein. Free ochratoxin, in turn, returns to the monoanionic form, which does not fluoresce.

Fig. 5A presents OTA fluorescence emission spectra at pH 7.4 depending on flurbiprofen concentration at the constant HSA concentration equal to 3 μM. After the addition of flurbiprofen to the OTA-HSA solution, a decrease in OTA fluorescence intensity is observed. This results from displacement of OTA from protein and return of OTA to the monoanionic form. A decrease in fluorescence intensity is directly proportional to the concentration of added flurbiprofen. Figure 5B presents how a relative fluorescence of the OTA-HSA complex changes at a wavelength of 448 nm in the function of flurbiprofen concentration. As the figure shows, the binding of OTA to albumins can be competitively replaced with some non-steroidal antiimflammatory drugs, such as flurbiprofen. This will accelerate the metabolism of OTA and contribute to faster excretion of this toxin from the organism. On the other hand, instead of chronic toxicity, the use of flurbiprofen may contribute to the occurrence of acute toxicity shortly after exposure to the toxin. Therefore, the use of this medicine must be connected with evaluation of the patient's condition and requires great caution.

CONCLUSION

The present study proved that ochratoxin A occurs in different ionic forms depending on the pH of the environment. At acidic pH it occurs in the monoanionic form, at alkaline pH in the dianionic form, whereas at physiological pH the occurrence of both forms is observed. Experimental results show that apart from the pH of the environment, the presence of HSA has also the essential effect on the ionic form of OTA. Since only the dianionic form of ochratoxin can be bound to albumin, the monoanionic form is transformed into the dianionic form under the influence of protein. Changing the ionic form of ochratoxin is connected with opening of its lactone ring. Results of the study show that flurbiprofen to a considerable extent affects a reduction in binding of ochratoxin to albumin. This non-steroidal antiinflammatory drug binds with albumin and is competitive for OTA binding sites.

Consequently, it shortens the time of ochratoxin presence in the vascular bed. On the one hand, thanks to binding OTA by plasma proteins, the toxin penetration into the target issues of the organism is delayed and reduced, while on the other, it prolongs the presence of this toxin in the vascular bed. Flurbiprofen and its possibly protective effect against the toxic effect of OTA suggests further studies of its application as an antidote.

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