VOLUME: 15 NO: 2 YEAR: 2018

ISSN: 1304-530X

TURKISH JOURNAL OF PHARMACEUTICAL SCIENCES

An Official Journal of the Turkish Pharmacists' Association, Academy of Pharmacy

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JOURNAL OF PHARMACEUTICAL SCIENCES

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Printing at: Özgün Ofset Ticaret Ltd. Şti.
Yeşilce Mah. Aytekin Sok. No: 21, 34418 4. Levent, İstanbul-Turkey
Phone: +90 280 00 09
Printing date: July 2018
ISSN: 1304-530X
E-ISSN: 2148-6247

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STARD checklist for the reporting of studies of diagnostic accuracy (Bossuyt PM, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, et al., for the STARD Group. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. Ann Intern Med 2003;138:40-4.) (http://www.stard-statement.org/);

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independently of the abbreviations used in the text. For original articles, the structured abstract should include the following sub-headings:

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Results: The detailed results of the study should be given and the statistical significance level should be indicated.

Conclusion: Should summarize the results of the study, the clinical applicability of the results should be defined, and the favorable and unfavorable aspects should be declared.

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Turk J Pharm Sci 2018;15(2):117-124
DOI: 10.4274/tjps.03522

ORIGINAL ARTICLE

Therapeutic Protection from Hepatic Injury and Chemical Constituents of *Buchanania angustifolia* Roxb

Buchanania angustifolia Roxb'un Kimyasal Bileşenleri ve Karaciğer Hasarına Karşı Terapötik Koruyucu Etkisi

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ABSTRACT

Objectives: The use of modern medicines in the treatment of diseases instead of traditional medicine is causing different adverse effects. Therefore, there is need to search new bioactive compounds to treat different diseases. *Buchanania angustifolia* has been used in traditional medicine in the treatment of different diseases. On the basis of folkloric information, *B. angustifolia* aerial parts extracts have been selected for their antioxidant and hepatoprotective potentiality and phytochemical constituents.

Materials and Methods: Qualitative phytochemical screening of *B. angustifolia* extracts revealed the presence of phytochemical constituents such as steroids, terpenoids, flavonoids, alkaloids, glycosides, tannins, carbohydrates, oils and amino acids. BA-1 and BA-2 compounds isolated from hydroalcoholic extract using chromatography and were identified as linolenic acid and mixture of stigmasterol and β-sitosterol.

Results: Antioxidant activity was performed on superoxide, hydroxyl and 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride free radicals. The *B. angustifolia* extracts showed dose-dependent activity on free radicals. The hydroalcoholic extract showed better activity. The extracts showed hepatoprotective activity on thioacetamide-induced liver intoxication in rats, and hydroalcoholic extract exhibited significant restoration of the altered biochemical parameters due thioacetamide-induced liver intoxication.

Conclusion: Among the tested extracts of *B. angustifolia,* hydroalcoholic extract showed higher antioxidant and hepatoprotective activity and stigmasterol and β-sitosterol were isolated. Further research is needed to evaluate other pharmacological activities and to isolate the other bioactive compounds from *B. angustifolia*.

Key words: *Buchanania angustifolia,* chemical constituents, antioxidant activity, thioacetamide, hepatoprotective activity

ÖZ

Amaç: Hastalıkların tedavisinde geleneksel tıp yerine modern ilaçların kullanımı farklı yan etkilere neden olabilmektedir. Bu nedenle, çeşitli hastalıkların tedavisinde kullanılmak üzere yeni biyoaktif bileşiklerin araştırılması gerekmektedir. *Buchanania angustifolia*, geleneksel tıpta farklı hastalıkların tedavisinde kullanılmaktadır. Bu çalışmada, folklorik bilgilere dayanılarak, *B. angustifolia* bitkisinin toprak üstü kısımlarının antioksidan ve hepatoprotektif etkilerinin ve fitokimyasal bileşenlerinin belirlenmesi amaçlanmıştır.

Gereç ve Yöntemler: *B. angustifolia* ekstreleri üzerinde yapılan kalitatif fitokimyasal analizler, ekstrelerde steroit, terpenoit, flavonoit, alkaloit, glikozit, tanen, karbonhidrat, yağ ve aminoasit yapısındaki bileşiklerin varlığını ortaya koymuştur. BA-1 ve BA-2 bileşikleri kromatografik teknikler kullanılarak sulu alkollü ekstreden izole edilmiş ve linolenik asit ile stigmasterol ve β-sitosterol karışımı olarak tanımlanmıştır.

Bulgular: Antioksidan aktivite, süperoksit, hidroksil ve 1-(2,6-dimetilfenoksi)-2-(3,4-dimetoksifeniletilamino) propan hidroklorit serbest radikalleri üzerinde gerçekleştirilmiştir. *B. angustifolia* ekstreleri, serbest radikallere karşı doz-bağımlı aktivite göstermiştir. Sulu alkollü ekstrenin daha iyi aktivite gösterdiği belirlenmiştir. Ekstreler sıçanlarda tiyoasetamid-nedenli karaciğer zehirlenmesinde hepatoprotektif etki göstermiş ve sulu alkollü ekstre, tiyoasetamid-nedenli karaciğer zehirlenmesine bağlı olarak değişen biyokimyasal parametreleri anlamlı derecede iyileştirmiştir.

Sonuç: *B. angustifolia*'nın test edilen ekstreleri arasında sulu alkollü ekstrenin daha yüksek antioksidan ve hepatoprotektif etkinlik gösterdiği belirlenmiştir ve stigmasterol ve β-sitosterol bileşikleri izole edilmiştir. *B. angustifolia*'nın diğer farmakolojik etkilerinin değerlendirilmesi ve başka biyoaktif bileşiklerinin izolasyonları için daha fazla araştırmaya ihtiyaç duyulmaktadır.

Anahtar kelimeler: *Buchanania angustifolia*, kimyasal bileşenler, antioksidan aktivite, tiyoasetamid, hepatoprotektif aktivite

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Received: 02.04.2017, **Accepted:** 08.06.2017

INTRODUCTION

Plants have been used as medicines since time immemorial around the world. The best known uses of plants in medicine were ayurveda in India, African traditional medicines, Unani medicine in Middle East countries, and in traditional Chinese medicine.[1-5] The major problems associated with traditional medicine are the lack of standardization, consistency, safety, and quality.[6] Advances in technology provided the perfection in medicine for treating the different diseases. Simultaneously, the use of modern medicine offered a new adverse effects with their long-term use and disease-causing microorganisms gaining resistance to drugs.[7,8] As such, people are turning back to traditional medicine because of their fewer adverse effects and the diversity of chemical entities in them for the treatment of different diseases. From this point of view, researchers are now focusing on the identification, evaluation, and standardization of the biologic activities of traditional medicinal plants[9] using advanced technology to isolate new bioactive molecules. The present work was performed on one of the traditional medicinal plants, *Buchanania angustifolia* Roxb (*B. angustifolia*), to isolate new bioactive molecules and to provide scientific evidence of its traditional use by Indian tribes for a wide range of ailments, including nutritional disorders, skin diseases, gravel, healing of wounds, rheumatic pain, tonic for sexual debility, and urinary problems.[10-12] *B. angustifolia* is mainly grown in dry deciduous forests and distributed around South India and Sri Lanka regions. However, to the best of our knowledge, the phytochemical constituents and biologic activities of *B. angustifolia* have not been reported.[13] Therefore, this study was planned to investigate the phytochemical constituents, antioxidant, and hepatoprotective potentials of hexane, ethyl acetate, and hydroalcoholic extracts of *B. angustifolia* aerial parts.

EXPERIMENTAL

Drug and chemicals

Chemicals used for the study were analytical grade. Silymarin, thioacetamide (TAA) and 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride (DPPH) were purchased from Sigma-Aldrich, St. Louis, USA, nitroblue tetrazolium was purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Riboflavin was purchased from Loba Chemie Pvt. Ltd., Mumbai, India. The kits for assessment of different biochemical parameters such as aspartate aminotransferase (AST/SGOT), alanine aminotransferase (ALT/SGPT), alkaline phosphatase (ALP), total bilirubin, and total protein were purchased from Span diagnostics Ltd., Gujarat, India. Folin-Ciocalteu reagent, bromocresol green were purchased from Sigma-Aldrich, St. Louis, USA.

Plant material collection and preparation of extracts

The plant material was collected from the Talakona forest region, Tirupathi, Andhra Pradesh, India, during the month of December, 2010 (AUCP/BGR/2010-431). Authentication of the plant was performed by Rtd. Prof. M. Venkaih, Department of Botany, Andhra University, Visakhapatnam. The plant material was dried under shade and powdered for separate extraction processes using maceration with hexane, ethyl acetate, and hydroalcoholic [ethanol (70% v/v)], and then concentrated to dryness under vacuum using rotavapour.

Preliminary phytochemical studies

The extracts of the *B. angustifolia* were subjected to different phytochemical tests for the identification of its phytochemical constituents, using standard procedures.[13]

Quantification of total phenolic content

The total phenolic content was determined using Folin-Ciocalteu reagent. The method is based on blue light absorption measurement due to the chemical reduction of tungsten and molybdenum oxides of Folin-Ciocalteu reagent, when combined with the compounds present in the extracts using colorimetry at 765 nm. The phenolic content in the extract was measured in gallic acid equivalents as mg/g, using a gallic acid calibration curve. The results are shown in mean values.[14,15]

Quantification of total alkaloid content

The plant extract (1 mg/mL) was dissolved in 2 N hydrochloric acid and the solution was filtered. The phosphate buffer's pH was neutralized to 0.1 N sodium hydroxide. One milliliter of extract solution, 5 mL of phosphate buffer, and 5 mL of bromocresol green solution were placed in separation and then the solution was mixed well. The complex formed in the solution was extract with chloroform. The absorbance of complex color in chloroform was measure at 470 nm. The experiment was performed in triplicate and results were reported in atropine equivalents. The results are presented as mean values.[14,16]

In vitro antioxidant activity

In vitro antioxidant activity was assessed use prepared extracts of *B. angustifolia* with dimethyl sulfoxide as a vehicle on superoxide, hydroxyl and DPPH free radicals.[14,17,18] The percentage inhibition and inhibition concentration $(IC)_{50}$ values were calculated.

Superoxide radical scavenging activity

The superoxide scavenging activities of the selected plant extracts were evaluated as per standard methods, using absorption of light at 560 nm, induction of superoxide free radical generation by riboflavin, and corresponding reduction by nitroblue tetrazolium.

Hydroxyl radical scavenging activity

The scavenging activity of selected plants extracts on hydroxyl radical was measured as per established methods. It was studied by the competition between deoxyribose and the extract's antioxidant molecules for hydroxyl radicals generated from the Fe^{+2}/EDTA/H_2O_2 system.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured as per methods. This method is based on measurements of color absorbance of alcoholic DPPH solution (blue color) after adding the antioxidant solution (extract/compound). If antioxidants are present in the test compound, the blue color turns to yellow due to DPPH.

Calculation of percentage inhibition

The percentage inhibition of superoxide production by the extract was calculated using the following formula:

Inhibitory ratio = $(A_0-A_1) \times 100 / A_0$

A_0: Absorbance of control, A_1: Absorbance of plant extract or/ and ascorbic acid

IC_{50} calculation form percentage inhibition

The optical density obtained with each concentration of the extract/ascorbic acid was plotted taking concentration on the X-axis and percentage inhibition on the Y-axis. The graph was extrapolated to find the 50% inhibition concentration of extract/ ascorbic acid.

Selection of animals

Healthy albino rats of either sex weighing between 180-250 g aged 60-90 days were used for the study. The rats were taken care of with standard light and humidity and with a supply of proper food and water.

Acute toxicity studies

The acute toxicity study was conducted for extracts of *B. angustifolia* extracts as per Organisation for Economic Co-operation and Development (OECD) guidelines 420 (OECD 2001) and regulations of the Institutional Animal Ethics Committee (Regd no: 516/01/A/CPCSEA). The albino mice of single sex, were selected into three groups, each consisting of 6 animals. They were maintained for one week before the experiment under room temperature and allowed free access to food and water. The animals were subjected to an acute toxicity study using each extract at a dose of 2000 mg/kg orally at regular intervals of time, i.e., 1, 2, 4, 8, 12 and 24 h. During this time, the animals were under observation to note different conditions such as skin changes, morbidity, aggressiveness, oral secretions, sensitivity to sound and pain, respiratory movements, and mortality.

Assessment of hepatoprotective activity of B. angustifolia

The selected plant extracts were tested for their hepatoprotective nature using TAA-induced liver toxicity in rats. For this experiment, animals were divided into twelve groups of 6 animals. Group I was treated with normal saline (vehicle) for one week through oral administration at 2 mL/kg body weight. Group II and Group III animals were treated with TAA as a 2% w/v solution in water on the first day at 50 mg/kg body weight subcutaneously (s.c.), then Group II was continuously treated with saline and Group III with silymarin at a dose of 25 mg/kg body weight p.o. for three weeks. Groups IV to XII were treated with TAA as a 2% w/v solution in water on the first day at 50 mg/kg body weight s.c., then Groups IV, V, and VI were treated with hydroalcoholic extract, Groups VII, VIII, and IX with ethyl acetate extract, and Groups X, XI, and XII were treated with hexane extract at a doses of 125, 250, 500 mg/kg body weight orally for three weeks. Animals of all groups were anaesthetized using chloroform 48 h after the final dose of extracts. Blood samples were collected from the animal groups from the retro-orbital plexus, then the samples were immediately centrifuged at 2400 rpm for quarters of an hour. Then, the clearly separated serum after centrifugation was used for measuring the different biochemical parameters using an auto analyzer with reagent kits.[19-21] All experimental procedures involving animals were conducted according to OECD guidelines and approved by the Institutional Animal Ethics Committee, Andhra University. Results were analyzed using Two-way ANOVA followed by the Bonferroni post-hoc test. All groups were compared with the silymarin group.

Isolation of phytoconstituents (Compounds)

There was very less phytochemical work reported on *B. angustifolia*. So, depends on availability of extracts after pharmacological study, hydroalcoholic extract of *B. angustifolia* was used for separation of compounds using column chromatography.

RESULTS

Qualitative phytochemical screening

Qualitative phytochemical screening of *B. angustifolia* extracts revealed the presence of different phytochemical constituents such as steroids, terpenoids, flavonoids, alkaloids, glycosides, tannins, carbohydrates, oils and amino acids. The extracts gave negative results for the quinines and saponins. All extracts of *B. angustifolia* revealed the presence of phenols, alkaloids, carbohydrates, steroids, terpenoids, and glycosides, and gave negative results to saponins. The hydroalcoholic and ethyl acetate extracts revealed the presence of flavonoids and tannins but the hexane extract gave negative results. The hexane and ethyl acetate extracts revealed the presence of a min amount of oil but hydroalcoholic extracts gave negative results. All extracts gave negative results to amino acids but the hydroalcoholic extracts give a weak positive result for the presence of amino acids (Table 1).

Table 1. Nature of phytoconstituents present in different extracts of *B. angustifolia* aerial parts

Phytochemical constituents	Extracts of *B. angustifolia* aerial parts		
	Hexane	Ethyl acetate	Hydroalcoholic
Phytosterols	+	+	++
Terpenoids	+	+	+
Glycosides	+	++	++
Saponins	-	-	-
Flavonoids	-	+	+
Tannins	-	+	+
Carbohydrates	+	+	+
Alkaloids	+	+	++
Amino acids	-	-	+
Oils	+	+	+
Phenols	+	+	++

+, ++: Present, -: Absent

Quantification of phenolic and alkaloid contents

The quantified phenolic contents of *B. angustifolia* extracts ranged from 13.85±1.22 to 34.10±2.62 mg/g. The hydroalcoholic extract had more phenolic content (34.10±2.62 mg/g) than other extracts. The quantitative alkaloid content ranged from 16.24±2.38 to 31.86±1.88 mg/g (Table 2).

Antioxidant activity

The hydroalcoholic, ethyl acetate and hexane extracts of *B. angustifolia* were found to possess concentration-dependent scavenging activity. The mean IC_{50} values for the hydroalcoholic, ethyl acetate, and hexane extracts of *B. angustifolia* on superoxide radical were found as 237±0.56 µg, 294±0.22 µg, and 450±0.43 µg, respectively, and for ascorbic acid, it was 54.4±1.1 µg (Figure 1, Table 3). The mean IC_{50} values for hydroxyl radicals were found as 265±0.82 µg, 231±0.62 µg, and 369±0.52 µg, respectively, and for ascorbic acid it was 68.00±1.3 µg (Figure 2, Table 3). The mean IC_{50} values of DPPH radicals were found as 206±0.18 µg, 272±0.14 µg, and 295±0.68 µg, respectively. The mean IC_{50} value of ascorbic acid was found as 22.0±0.5 µg (Figure 3, Table 3). Among all the tested extracts, better free radical scavenging activity was found for the hydroalcoholic extract of *B. angustifolia* aerial parts.

Acute toxicity studies

There were no visible signs of toxicity and mortality, and no behavioral changes such as alertness, motor activity, breathlessness, restlessness, diarrhea, tremor, convulsion, and coma were observed at the administered doses. The animals were physically active and no deaths were recorded, even at doses of up to 2000 mg/kg body weight. Hence, all the tested extracts were considered as safe and nontoxic.

Table 2. Total phenolic and alkaloid contents (mg/gm) of *B. angustifolia* aerial parts' extracts

S. No	Name of the extract	Total phenolic content (mg/gm)	Total alkaloid content (mg/gm)
1	Hexane	13.85±1.22	16.24±2.38
2	Ethyl acetate	26.28±0.66	22.40±1.36
3	Hydroalcoholic (ethanol 70%)	34.10±2.62	31.86±1.88

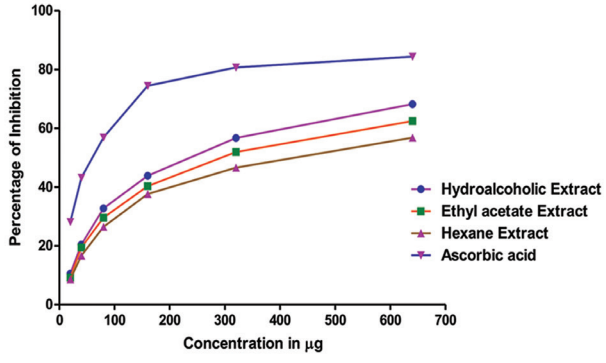

Figure 1. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on superoxide radical

Hepatoprotective activity

We examined the *B. angustifolia* aerial parts at three dose levels, 125 mg/kg, 250 mg/kg, and 500 mg/kg, which was assessed by measuring liver-related biochemical parameters (AST, ALT, ALP, total serum bilirubin, and total protein) levels for their hepatoprotective nature using TAA-induced hepatotoxicity in rats.

Group I, which was treated with vehicle, showed no significant changes in liver enzyme biomarker levels (AST, ALT, ALP, total bilirubin, and total protein). Group II was treated TAA, there were significant changes in levels of biomarker enzymes. The animals of Group III were administered TAA and then silymarin (Table 4). There were significant changes in biomarker enzymes levels compared with Group II rats' enzymes levels and the percentage protection offered by the silymarin against the changes in AST, ALT, ALP, total bilirubin, and total protein levels were 96.24%, 95.25%, 93.90%, 97.83%, and 96.14%, respectively.

The percentage protection produced by the hydroalcoholic extract (Groups IV, V and VI) on the enhancement of AST, ALT, ALP, total bilirubin, and total protein levels were 45.45%, 45.01%, 38.35%, 35.38%, and 32.87%; 57.04%, 58.40%, 51.79%, 48.21%, and 46.05%; 76.39%, 78.77%, 69.45%, 62.74%, and 65.81%, respectively.

The percentage protection produced by the ethyl acetate extracts (Groups VII, VIII, and IX) the enhancement of AST,

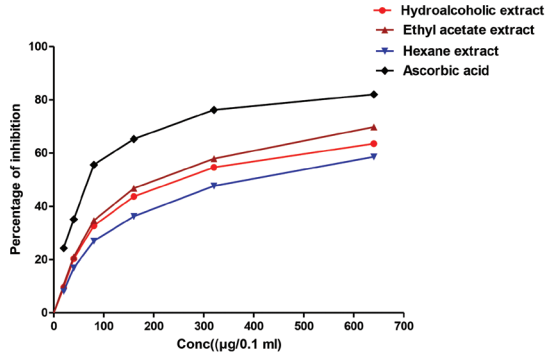

Figure 2. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on hydroxyl radical

Table 3. Fifty percent IC_{50} of different extracts of *B. angustifolia* against superoxide, hydroxyl and DPPH radicals

Name of the extract	50% IC_{50} (µg/mL)		
	Superoxide radical	Hydroxyl radical	DPPH radical
Hydroalcoholic	237±0.56	265±0.82	206±0.18
Ethyl acetate	294±0.22	231±0.62	272±0.14
Hexane	450±0.43	369±0.52	295±0.68
Ascorbic acid	54.4±1.1	68.00±1.3	22.0±0.5

DPPH: 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride, IC_{50}: Inhibition concentration

ALT, ALP, total bilirubin, and total protein levels were 43.03%, 41.03%, 36.08%, 29.40%, and 26.94%; 54.20%, 55.41%, 49.57%, 45.64%, and 47.36%; 66.36%, 67.38%, 60.04%, 59.32%, and 61.20%, respectively.

The percentage protection produced by the hexane extracts (Groups X, XI, and XII) on the enhancement of AST, ALT, ALP, total bilirubin, and total protein levels were 37.20%, 37.18%, 30.55%, 25.98%, and 24.97%; 48.29%, 47.72%, 42.43%, 40.51%, and 40.78%; 58.75%, 59.83%, 53.60%, 51.62%, and 52.64%, respectively.

The decrease in the AST, ALT, ALP and total bilirubin levels, and increase in levels of protein to normal, and percentage protection produced by the higher doses of the extracts were comparable to those of silymarin. Among all the extracts, the hydroalcoholic extracts of selected plants showed better activity (Table 5).

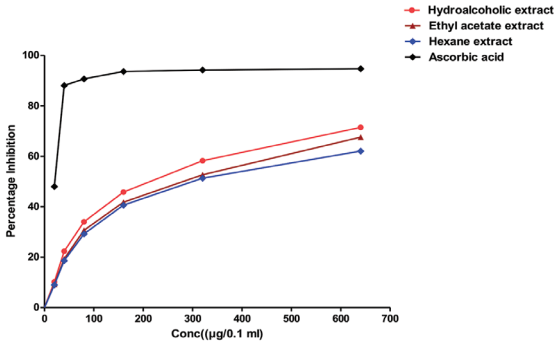

Figure 3. Concentration dependent percentage inhibition of different extracts of *B. angustifolia* on 1-(2,6-dimethylphenoxy)-2-(3,4-dimethoxyphenylethylamino) propane hydrochloride radical

Isolation of compounds

The selected plant extracts (hexane, ethyl acetate, and hydroalcoholic) on thin-layer chromatography showed different spots with different retention factor values, but on the basis of biologic activities (antioxidant and hepatoprotective), the hydroalcoholic extract of *B. angustifolia* was used for the separation of compounds using column chromatography.

Column chromatography was performed using a standard procedure. Silica gel (Qualigens), 60-120 mesh was used as an absorbent for column chromatography. The column was eluted using hexane, hexane-ethyl acetate, ethyl acetate, and ethyl acetate-methanol mixtures by gradient. BA-1 and BA-2 compounds were isolated in the study.

Structure elucidation and characterization of compound BA-1

Compound BA-1 was isolated in the combination of hexane and ethyl acetate (95:05) in the form of colorless oil. The proton nuclear magnetic resonance (1H-NMR) spectrum (Figure 4) of compound BA-1 in acetone exhibited signals due to olefinic

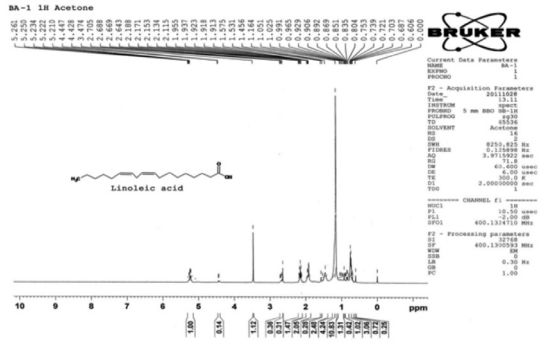

Figure 4. The proton nuclear magnetic resonance spectrum of compound BA-1

Table 4. Enzymes levels due to the effect of *B. angustifolia* extracts at different doses on TAA-induced liver toxicity in rats

Name of extract/compound	Quantity of extract (mg/kg b.w.)	Enzymes levels of TAA-induced hepatotoxicity				
		AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mg/dL)	Total protein (g/dL)
Normal (drug vehicle)	2 mL	96.17±6.97	56±3.58	217.5±2.59	0.17±0.01	7.40±0.46
TAA	50 mg	330.5±6.53	173±5.22	548.67±21.60	2.123±0.27	4.87±0.78
Silymarin	25 mg	87±2.83	50.17±4.96	196.00±5.10	0.13±0.05	7.50±0.37
Hydroalcoholic	125 mg	224±3.03	120.3±4.32	421.67±6.65	1.43±0.29	5.7±0.37
	250 mg	196.8±2.04	104.67±3.01	377.17±3.13	1.18±0.27	6.03±0.29
	500 mg	151.5±2.66	80.8±2.32	318.67±2.58	0.9±0.18	6.53±0.32
Ethylacetate	125 mg	229.67±3.72	125±4.00	429.17±2.32	1.55±0.23	5.55±0.22
	250 mg	203.5±4.28	108.17±2.23	384.5±4.97	1.23±0.24	6.07±0.18
	500 mg	175±2.83	94.17±3.71	349.8±2.14	0.97±0.18	6.42±0.35
Hexane	125 mg	243.3±4.46	129.5±2.43	447.5±3.21	1.62±0.27	5.5±0.37
	250 mg	217.3±3.50	117.17±2.23	408.17±2.79	1.33±0.20	5.9±0.41
	500 mg	192.8±2.64	103±2.19	371.17±2.40	1.12±0.17	6.2±0.34

TAA: Thioacetamide, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase

Table 5. Percentage (%) protection of enzymes levels due to the effect of *B. angustifolia* extracts at different doses on TAA-induced liver toxicity in rats

Name of extract	Quantity of extract	Percentage protection on enzymes levels of TAA-induced hepatotoxicity				
		AST (U/L)	ALT (U/L)	ALP (U/L)	Total bilirubin (mg/dL)	Total protein (g/dL)
Silymarin	50 mg/kg b.w.	96.24	95.25	93.90	97.83	96.14
Hydroalcoholic	125 mg/kg b.w.	45.45**	45.01**	38.35**	35.38**	32.87**
	250 mg/kg b.w.	57.04**	58.40**	51.79**	48.21*	46.05*
	500 mg/kg b.w.	76.39**	78.77***	69.45**	62.74**	65.81***
Ethylacetate	125 mg/kg b.w.	43.03*	41.03**	36.08*	29.40*	26.94*
	250 mg/kg b.w.	54.20*	55.41**	49.57**	45.64*	47.36**
	500 mg/kg b.w.	66.36**	67.38***	60.04**	59.32**	61.20**
Hexane	125 mg/kg b.w.	37.20*	37.18*	30.55*	25.98*	24.97*
	250 mg/kg b.w.	48.29*	47.72**	42.43**	40.51*	40.78**
	500 mg/kg b.w.	58.75**	59.83***	53.60**	51.62**	52.64***

TAA: Thioacetamide, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase
***p<0.001,**p<0.01, *p<0.05

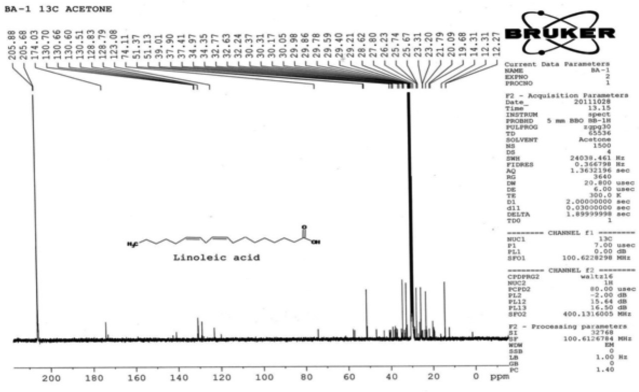

Figure 5. 13C-nuclear magnetic resonance spectrum of compound BA-1

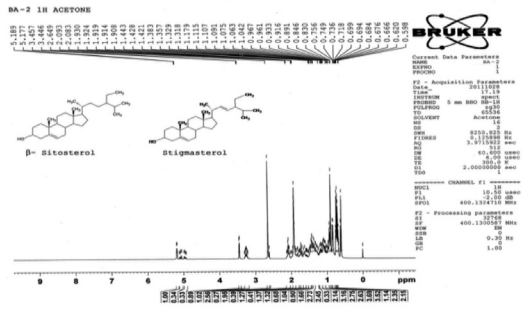

Figure 6. The proton nuclear magnetic resonance spectrum of compound BA-2

protons at δ 5.3~5.4, triplet methylene protons at δ2.8, 11 methylene protons at δ2.3, 2.0, 1.6 and 1.3, and methyl protons at δ.8. These signals were well matched to corresponding signals of linoleic acid, suggesting that this compound was linoleic acid or an unsaturated fatty acid. In the electrospray ionisation-mass measurement, its molecular weight was determined as 280 through a quasi-molecular ion peak at m/z 279 [M-H]-in the negative mode. This molecular weight was consistent with linoleic acid. Therefore, compound BA-1 was identified as linoleic acid. The introduction of a second double-bond to give linoleic acid or methyl linoleate (methyl 9(*Z*),12(*Z*)-octadecadienoate) gives rise to a peak at 2.8 ppm caused by the *bis*-allylic protons located at C11. The theoretical integration value of the olefinic protons increases to four, whereas that of the large methylene peak decreases further to 14.

Generally, many effects observed in 1H-NMR are also found in 13C-NMR (Figure 5). For example, the methyl and methylene signals are up field in the spectrum, and signals of olefinic carbons are farther downfield. The number and nature of double-bonds affects the chemical shifts, as do the proximity of multiple double-bonds to each other and the presence of functional groups.

Structural elucidation of compound BA-2

Compound BA-2 was isolated as a white solid with colorless needles, a mixture containing two sterolic compounds from the hexane soluble in combination of hexane and ethyl acetate (90:10). The mixture gave a positive color reaction with sulphuric acid indicating the sterolic nature. The molecular formula $C_{29}H_{48}O$ (stigmasterol) and $C_{29}H_{50}O$ (β-sitosterol) were established showing molecular ion peak [M]+ at *m/z* 412.3920, $[M]^+$ at 414, respectively, from the previous data as explained earlier. The 1H and 13C-NMR data are provided in Figures 6 and 7.

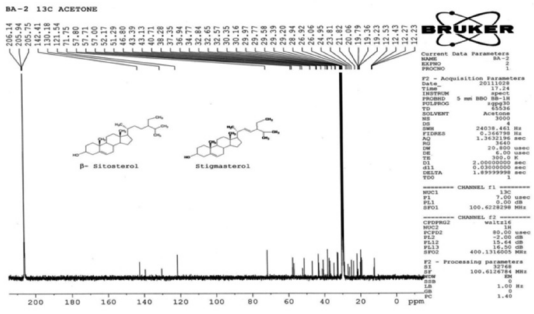

Figure 7. 13C-nuclear magnetic resonance spectrum of compound BA-2

DISCUSSION

Modern medicine significantly decreased mortality from different diseases compared with before the mid-nineteenth century. However, these developed medicines that cure diseases cause different adverse effects with long-term use, and their injudicious use has led to the development of drug-resistant diseases.[22,23] Medicinal plants have been used for the prevention and treatment of diseases for a long time.[24,25] There is a necessity to identify new bioactive molecules or drugs through science and technology from natural sources including medicinal plants, which are safer with no adverse effects and low cost.[24] In recent years, many researchers have reported on new drugs and validated traditional medicinal plants.[25] The results of the present study provide scientific evidence for the traditional use of *B. angustifolia.*[10-12] The qualitative phytochemical analysis and quantification of total phenolic and alkaloid contents and biologic activities were studied for *B. angustifolia*. Qualitative phytochemical screening of different extracts revealed the presence of different phytochemical constituents such as steroids, terpenoids, phenols, flavonoids, alkaloids, glycosides, phenols, tannins, carbohydrates, oils, and amino acids. The extracts on qualitative analysis on phenolic and alkaloid contents of hydroalcoholic extract showed greater content in it compared with other extracts.

Many investigations have reported that medicinal plants contain a wide variety of natural bioactive compounds that possess biologic activities.[26-30] Accordingly, the extracts were tested for their antioxidant capacity and hepatoprotective activity on TAA-induced liver toxicity. Many investigations explained the positive correlation between oxidative stress and liver toxicity.[31] The extracts of *B. angustifolia* showed a dose-dependent percentage inhibition on tested free radicals, but it was lower activity compared with ascorbic acid; as the concentration increases, the percentage inhibition may increase. The extracts reduced the formation of superoxide free radicals in the tested method; superoxide radical is the main free radical for oxidative stress by being involved in the formation of other free radicals.[32] At the same time, the extracts also showed hepatoprotective activity on TAA-induced liver toxicity in rats.[19-21] TAA-induced liver toxicity was established as the method to evaluate natural products for hepatoprotective activity and its mechanism involved for liver toxicity by damaging the mitochondria and dysfunction of intracellular organelles of hepatic cells. This ultimately leads to an increase in the amount of bile acid, greater amounts of which generally promote liver damage by oxidative stress[31] and often elevate liver biomarker enzymes in the body i.e. AST, ALT, ALP, total bilirubin, and total protein. Thus, biomarker enzymes were analyzed in the present study. The results of the hepatoprotective activity in the present study indicate that *B. angustifolia e*xtracts possesses moderate hepatoprotective activity compared with the standard drug silymarin. The extracts were significantly ($p<0.05$) normalized the elevated biomarker enzymes levels compared with the toxic group (Group II). As mentioned earlier, there may be a relationship between the antioxidant activity and hepatoprotective activity of *B. angustifolia extracts.* Among all the extracts, the hydroalcoholic extract showed more activities and higher phenolic and alkaloid contents. From this point of view, we tried to isolate new bioactive molecules/compounds using hydroalcoholic extract using column chromatography, but unfortunately, known compounds i.e. mixtures of stigmasterol and β-sitosterol and linolenic acid were isolated. However, these compounds are reported from this plant species for the first time. The isolated compounds, stigmasterol and β-sitosterol were isolated from *B. angustifolia* are may be responsible for the activities of selected plants because there were some earlier reports of different biologic activities of these compounds[33-39] or some other unknown compounds may be acting individually or may be synergistically.

CONCLUSIONS

The results of the present study provide evidence about traditional medicinal use of *B. angustifolia,* which is a traditional medicinal plant in India, and isolated known compounds stigamsterol and β-sitosterol from hydroalcoholic extract, were first reported from this species and maybe from this genus. Further research is needed to evaluate more pharmacologic activities of *B. angustifolia* and to isolate the more bioactive compounds through chromatographic techniques. Standardization of these isolates can be carried out by obtaining a chemical fingerprint/profile.

ACKNOWLEDGEMENTS

The authors are acknowledged to the authorities of Andhra University in providing the necessary facilities and also to the Rajiv Gandhi National Fellowship (RGNF)-UGC [No: F. 16-1577(SC)/2010 (SA-III)], New Delhi for financial support which enabled me to successfully complete the research.

Conflicts of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):125-129
DOI: 10.4274/tjps.62533

ORIGINAL ARTICLE

A Comparative Study on the *in vitro* Antioxidant and Antimicrobial Potentials of Three Endemic *Ononis* L. Species from Turkey

Türkiye'den Üç Endemik *Ononis* L. Türünün *in vitro* Antioksidan ve Antimikrobiyal Potansiyelleri Üzerine Karşılaştırmalı Bir Çalışma

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ABSTRACT

Objectives: In this study, the antioxidant capacity, antimicrobial activity and phenolic contents of aerial parts and roots extracts of three endemic *Ononis* L. (Leguminosae) species (*O. sessilifolia* Bornm., *O. basiadnata* Hub. & Mor., *O. macrosperma* Hub. & Mor.) were investigated for the first time.

Materials and Methods: The phenolic contents of the extracts [water and ethanol (EtOH)] and fractions [dichloromethane, EtOAc and *n*-butanol] were determined using Folin-Ciocalteu's phenol reagent. Also, their antioxidant capacities were studied using qualitative $DPPH^{\cdot}$ (1,1-diphenyl-2-picrylhdrazyl radical) and TBA assays. The antimicrobial activity of these extracts and fractions compared with standard antibiotics were studied using disc diffusion assays against various Gram-positive and Gram-negative bacteria and fungi.

Results: The total phenolic contents of the water extracts were found to range between 14.78-80.33 mg/g, and the EtOH extracts ranged from 67.19-145.33 mg/g. EtOAc fractions of the three species were rich in terms of total phenolic contents when compared with other extracts (242.56-620.89 mg/g). The most significant results in the TBA assays were obtained in EtOH extracts of *O. macrosperma* (IC_{50}=0.13±0.17 µg/mL), *O. sessilifolia* (IC_{50}=1.41±0.58 µg/mL) and root (IC_{50}=1.96±0.39 µg/mL).

Conclusion: EtOAc fractions rich in phenolic content were also found to be the most effective in antioxidant activity assays. Although all water extracts had no antimicrobial activity, EtOH extracts and *n*-butanol fractions showed generally moderate activity against bacteria. Some EtOAc fractions except for *O. sessilifolia* showed less activity against *Escherichia coli*, *Staphylococcus aureus*, *MRSA* and *Candida albicans*.

Key words: *Ononis sessilifolia*, *Ononis basiadnata*, *Ononis macrosperma*, antioxidant capacity, antimicrobial activity, endemic

ÖZ

Amaç: Bu çalışmada üç endemik *Ononis* L. (Leguminosae) türünün (*O. sessilifolia* Bornm., *O. basiadnata* Hub. & Mor., *O. macrosperma* Hub. & Mor.) antimikrobiyal aktivite, antioksidan kapasite ve fenolik içerikleri ilk kez araştırılmıştır.

Gereç ve Yöntemler: Ekstrelerin [su ve etanol (EtOH)] ve fraksiyonların [diklorometan, etil astetat (EtOAc) ve *n*-butanol] fenolik içerikleri Folin-Ciocalteu'nun fenol reaktifi kullanılarak belirlendi. Ayrıca, antioksidan kapasiteleri kalitatif $DPPH^{\cdot}$ (1,1-diphenyl-2-picrylhdrazyl radical) ve TBA deneyleri ile çalışıldı. Ekstre ve fraksiyonların antimikrobiyal aktivitesi disk difüzyon tekniği kullanılarak standart antibiyotiklere kıyaslanarak çeşitli Gram-pozitif, Gram-negatif ve mantara karşı çalışıldı.

Bulgular: Su ekstrelerinin toplam fenol içerikleri 14.78-80.33 mg/g aralığında değişirken, EtOH ekstreleri 67.19-145.33 mg/g aralığında bulunmuştur. Üç türün EtOAc fraksiyonları, diğer ekstrelerle (242.56-620.89 mg/g) karşılaştırıldığında toplam fenol içeriği bakımından zengindir. TBA metodundaki en önemli sonuçlar, *O. macrosperma* herba (IC_{50}=0.13±0.17 µg/mL), *O. sessilifolia* herba (IC_{50}=1.41±0.58 µg/mL) ve kök (IC_{50}=1.96±0,39 µg/mL) EtOH ekstraktlarında elde edildi.

Sonuç: Fenolik içerik bakımından zengin olan elilasetat fraksiyonları, antioksidan aktivite deneylerinde de en yüksek etkili olarak tespit edilmiştir. Su ekstrelerinin antimikrobiyal aktivitesi olmamasına rağmen, türlerin EtOH ekstreleri ve *n*-butanol fraksiyonları, bakterilere karşı genellikle orta düzeyde etkinlik gösterdi. *O. sessilifolia* dışında bazı EtOAc fraksiyonları, *Escherichia coli*, *Staphylococcus aureus*, *MRSA* ve *Candida albicans*'a karşı daha az aktivite gösterdi.

Anahtar kelimeler: *Ononis sessilifolia*, *Ononis basiadnata*, *Ononis macrosperma*, antioksidan kapasite, antimikrobiyal aktivite, endemik

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Received: 31.03.2017, **Accepted:** 22.06.2017

INTRODUCTION

Antioxidants have been recognized as potential therapeutics for preventing different human diseases (e.g., cancer, aging, cardiovascular diseases, asthma, acute central nervous system injury, neurodegenerative disease, and malaria).[1,2] Medicinal plants and herbs have played an important role in the health care of ancient and modern cultures. They are promising as natural antioxidant sources.[3,4] The number of studies on new natural antioxidants with plant origin is increasing.[5] *Ononis* L. genus (Leguminosae) is represented by 18 species, 4 of which are endemic to Turkey.[6] The *Ononis* species has various pharmacologic properties, such as antioxidant, aperient, diuretic, antimicrobial, analgesic, antiviral, cytotoxic, anti-inflammatory and anti-diarrheal activities. In Turkish folk medicine, *O. spinosa* L. has been used for the urinary tract, kidney stones, inflammatory diseases, wounds healing, and skin disorders.[1,7-9] Süntar et al.[10] reported that water and ethanolic extracts of the herb *O. macrosperma* demonstrated the highest activity in both wound models and anti-inflammatory activity. In previous studies, some *Ononis* species were found to contain different component groups such as isoflavone, triterpene, sterol, pterocarpane, and resorcinol derivatives, flavonoids, isocoumarins, and hydroxycinnamic acids.[1,11-13] A $DPPH^{\cdot}$ (1,1-diphenyl-2-picrylhdrazyl radical) radical is a stable radical with maximum absorbance at 517 nm, and when reduced to hydrazine derivatives by electron and hydrogen atom transfer from substances with antioxidant properties, its absorbance is decreased.[14] Lipid peroxidation is a chain reaction that causes the deterioration of biologic systems, and is the accumulative effect of reactive oxygen species. Reactive free radicals start the reaction by the deletion of allylic hydrogen atoms from the methylene group of unsaturated fatty acids.[15] Thiobarbituric acid (TBA) and DPPH methods have been used to evaluate the antioxidant capacities of the plant extracts/component.[16] Medicinal plants represent potential sources of natural antioxidant and antimicrobial agents for food and medicinal purposes. The purpose of this investigation was to study antioxidant activities using TBA and DPPH [thin layer chromatography (TLC) screening], determine phenolic contents using spectrophotometry, and the antimicrobial activity with disc diffusion assays of herb and root extracts of three *Ononis* species. This is the first study on the antioxidant capacity and antimicrobial activities of the three *Ononis* species.

MATERIALS AND METHODS

Plant materials

Ononis species were gathered from different provinces of Turkey in their natural habitats. Voucher specimens were stored in the Herbarium of the Faculty of Pharmacy at the University of Ankara, Turkey (AEF). *O. sessilifolia* was collected from the Çamardı county of Niğde in June 2007 (AEF 23979); *O. basiadnata* was collected from the Gülnar county of İçel in June 2007 (AEF 23968); and *O. macrosperma* was collected from the Elmalı county of Antalya in May 2008 (AEF 24698).

Extraction of plants

The herb and root of the *O. basiadnata* and *O. sessilifolia* and the herb of *O. macrosperma* were powdered and then 50 g of the herbs and roots were macerated separately with 500 mL of water for 5 h at 60°C. Afterwards, the water extracts were filtered, frozen, and lyophilized. One hundred grams of plant material was macerated with ethanol (EtOH) for 5 h at 50°C. The extracts were then filtered and evaporated until dry. Ethanolic extracts were dispersed in methanol: water (1:9), partitioned with dichloromethane (DCM), ethyl acetate (EtOAc) and *n*-butanol (BuOH). Afterward, the fractions were evaporated until dry.

Determination of total phenolic content

The total phenolic contents of extracts were evaluated using the Folin-Ciocalteu assay as gallic acid equivalents (GAE).[17]

Antioxidant capacities of extracts

DPPH test

The antioxidant capacity of extracts (EtOH and water extracts; DCM, EtOAc and BuOH fractions) were evaluated with rapid TLC screening.[18]

TBA test (measurement of malondialdehyde value)

The amount of malondialdehyde formed in the reaction mixture was determined using the TBA reagent spectrophotometrically.[18]

Antimicrobial activity

Test microorganisms

The test microorganisms used in the experiment were Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* (*MRSA*) (clinical isolate), *Bacillus subtilis* ATCC 25923); Gram-negative bacteria (*Escherichia coli* ATCC 25922) and fungus: (*Candida albicans* ATCC 10231).

Ampicillin sulbactam (20 µg), ciprofloxacin (5 µg), fluconazole (25 µg) and cefotaxime (30 µg) were used as control drugs. ATCC strains were obtained from the culture collection of the Refik Saydam Health Institution of Health Ministry, Ankara.

Media

Mueller-Hinton agar (Difco, Detroit, MI, USA) was used for bacteria, and MHA supplemented with 2% glucose and 0.5 µg/mL methylene blue was used for *C. albicans*.

Disc diffusion assay

Antimicrobial activities of the extracts and fractions were evaluated by using the disc diffusion technique.[19,20]

RESULTS

Phenolic compounds are known as a main class of active compounds determined by Folin-Ciocalteu assay. The highest total phenolic contents of all extracts and fractions of the three species were determined in the EtOAc fractions. The results of total phenolic contents of extracts and fractions are shown in Table 1.

When all DCM fractions and EtOAc fractions, excluding *O. basiadnata* root, were compared using propyl gallate, they were

Table 1. Total phenolic contents of the extracts and fractions of *Ononis* species

Species	Total phenolic contents ($mg_{gallic\ acid}/g_{extr}$) ± SD*				
	Water extracts	EtOH extracts	DCM fraction	EtOAc fraction	BuOH fraction
O. sessilifolia herb (OSH)	37.19±1.58	132.01±4.16	208.11±3.93	413.67±5.50	124.77±0.79
O. sessilifolia root (OSR)	14.78±0.79	145.33±1.57	221.44±2.36	327.08±0.79	131.44±2.36
O. basiadnata herb (OBH)	80.33±0.79	111.44±0.79	105.33±4.16	620.89±12.57	89.77±4.71
O. basiadnata root (OBR)	20.15±0.64	91.17±5.55	119.22±2.36	242.56±4.84	88.11±0.79
O. macrosperma herb (OMH)	46.17±3.78	67.19±0.64	63.67±4.21	467.03±3.93	156.44±4.71

*SD: Standard deviation, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

Table 2. Antioxidant capacities of extracts and fractions of *Ononis* species in TBA test

Species	IC_{50} value (µg/mL) ± SD*				
	Water ext.	EtOH ext.	DCM frac.	EtOAc frac.	BuOH frac.
OSH	NE*	1.41±0.58	14.38±1.32	51.18±3.31	NE
OSR	>1000±8.55	1.96±0.39	26.03±0.24	146.35±2.73	NE
OBH	>1000±3.11	24.19±2.21	49.73±1.41	532.01±5.58	NE
OBR	>1000±7.99	15.57±0.95	26.06±1.19	3.10±1.14	138.93±2.26
OMH	NE	0.13±0.17	>1000±2.85	61.56±3.61	570.41±7.14
Propyl gallate	3.72±1.6				

*Non-effective; SD: Standard deviation, OSH: *O. sessilifolia herb*, OSR: *O. sessilifolia root*, OBH: *O. basiadnata herb*, OBR: *O. basiadnata root*, OMH: *O. macrosperma herb*, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: n-butanol

Table 3. Results of the antimicrobial activity of *O. sessilifolia* herb (OSH) and root (OSR) extracts (inhibition zones in mm)

Extracts/Drugs OSA	*S. aureus* ATCC 25923	*MRSA* isolate	*B. subtilis* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
OSH EtOH ext.	10	12	-	7	13
OSR EtOH ext.	-	5	-	-	11
OSH DCM	-	-	-	-	-
OSR DCM	-	-	-	-	-
OSH EtOAc	-	-	-	15	-
OSR EtOAc	-	-	-	-	-
OSH BuOH	11	15	15	17	12
OSR BuOH	14	11	10	16	17
OSH water ext.	-	-	-	-	-
OSR water ext.	-	-	-	-	-

OSH: *O. sessilifolia* herb, OSR: *O. sessilifolia* root, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: *n*-butanol

shown to have a high radical scavenging effect with qualitative DPPH. Yellow zones on a purple ground were marked for the DCM and EtOAc fractions of all species. In DPPH test, EtOH extracts were generally active from the water extract, especially the root and herb extracts of *O. sessilifolia* (Figure 1).

The most significant results in the TBA method were obtained in EtOH extracts of *O. macrosperma* herb (OMH) (IC_{50}=0.13±0.17 µg/mL), *O. sessilifolia* herb (IC_{50}=1.41±0.58 µg/mL) and root

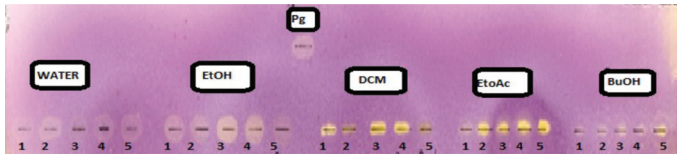

Figure 1. Antioxidant capacity by qualitative DPPH test on TLC of *Ononis* species

1: *O. basiadnata* root extract, 2: *O. basiadnata* herb extract, 3. *O. sessilifolia* root extract, 4: *O. sessilifolia* herb extract, 5: *O. macrosperma* herb extract, Pg: Propyl gallate

(IC_{50}=1.96±0.39 µg/mL) (Table 2). A survey of the published literature shows that the antioxidant activity of *O. sessilifolia*, *O. basiadnata* and *O. macrosperma* has not been subjected to research so far.

In our antimicrobial activity studies, the extracts and fractions of three endemic *Ononis* species were examined against various bacteria and fungi. First, EtOH extracts were prepared, and then extracted with DCM, EtOAc and BuOH. The antimicrobial activity of the water extract was also examined. Water extracts of herb and root parts had no antimicrobial activity against Gram (-), Gram (+) bacteria and yeast. All of the BuOH extracts showed moderate activity compared with the standards. Some EtOAc fractions also demonstrated less activity against *E. coli*, *S. aureus*, *MRSA* and *C. albicans*. Apart from OMH, other DCM extracts showed no activity against Gram (+), Gram (-) bacteria and fungi. All EtOH extracts showed less activity against some bacteria. In addition to this, they showed moderate activity against *C. albicans* according to fluconazole (Table 3, 4, 5 ,6).

DISCUSSION

In the literature, there are some studies on the antioxidant activities and phenolic contents of other *Ononis* species. Leaf methanolic extract of *O. natrix* has significant total phenolic content (51 mg GAE/g DW) and flavonoid content (14.76 CE/g DW).[21] The antioxidant activity and total phenolic contents of *O. natrix* used in folk medicine in Jordanian were identified as follows: according to antioxidant capacity results, the aqueous extract has 82.0±1.5 µmol TE/g, methanolic extract has 76.7±2.0 µmol TE/g dry weight; in total phenolic content, the aqueous extract has 16.9±0.4 mg GAE/g, methanolic extract has 21.1±0.7 mg GAE/g dry weight.[9] In another study, *O. spinosa* root infusion was evaluated in both DPPH inhibition (20.5±0.8%) and total phenolic content 3.09±0.01 mg GAE/g extract.[22] Although the ethanolic extract of *O. spinosa* indicated concentration-dependent superoxide anion radical scavenging capacity (IC_{50}=1.35 mg/mL), the extract showed no concentration-dependent inhibitory effect on lipid peroxidation.[23] Unlike the present study, in our lipid peroxidation experiment, significant results were obtained (Table 2).

Table 4. Results of the antimicrobial activity of *O. basiadnata* herb (OBH) and root (OBR) extracts (inhibition zones in mm)

Extracts/Drugs OBA	*S. aureus* ATCC 25923	*MRSA* isolate	*B. subtilis* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
OBH EtOH ext.	11	-	12	10	11
OBR EtOH ext.	10	7	-	14	10
OBH DCM	-	-	-	-	-
OBR DCM	-	-	-	-	-
OBH EtOAc	10	11	-	-	7
OBR EtOAc	-	-	-	14	-
OBH BuOH	12	12	14	14	-
OBR BuOH	11	14	17	17	14
OBH water ext.	-	-	-	-	-
OBR water ext.	-	-	-	-	-

OSR: *O. sessilifolia* root, OBH: *O. basiadnata* herb, OBR: *O. basiadnata* root, OMH: *O. macrosperma* herb, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: *n*-butanol

Table 5. Results of the antimicrobial activity of *O. macrosperma* herb (OMH) extracts (inhibition zones in mm)

Extracts/ Drugs OMH	*S. aureus* ATCC 25923	*MRSA* İsolate	*B. subtilis* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
EtOH ext.	10	9	-	-	16
DCM frac.	12	-	-	7	-
EtOAc fract.	-	10	-	-	-
BuOH frac.	12	15	-	16	13
Water ext.	-	-	-	-	-

OMH: *O. macrosperma* herb, EtOH: Ethanol, DCM: Dichloromethane, EtOAc: Ethyl acetate, BuOH: *n*-butanol

Table 6. Inhibition zones of standard antibiotics (inhibition zones in mm)

Reference Substances	*S. aureus* ATCC 25923	*MRSA* isolate	*B. subtilis* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 10231
Ampicillin sulbactam	30	-	21	-	-
Ciprofloxacin	-	27	-	-	-
Fluconazole	-	-	-	-	32
Cefotaxime	-	-	-	25	-

The present study shows that the three *Ononis* species contained phenolic compounds, which inhibit the oxidation of lipids by donating hydrogen atoms to scavenge free radicals.[24] Phenolic compounds have been shown to be more effective antioxidants than vitamin A and C.[25] Our results showed that there seemed to be good compatibility between the phenolic content and antioxidant capacity of the extracts because the EtOAc fractions with a higher phenolic content showed higher DPPH radical scavenging capacity.

According to previous studies, the BuOH extracts of *O. spinosa* (4 mg/disc) had moderate antifungal activity against *Aspergillus flavus*, *Fusarium moniliforme* and *C. albicans* in comparison with miconazole nitrate at 40 µg/disc. Petroleum benzene, EtOH, and water extracts showed high activity against Gram (+) and Gram (-) bacteria.[26] In another study, EtOH extract of *O. spinosa* demonstrated significant activity against Gram-positive (*E. coli* and *P. aeruginosa*), Gram-negative (*S. aureus*) bacteria and fungi (*C. albicans*).[27] In our study, similar results were obtained, EtOH extracts and BuOH fractions of all species showed generally high activity against Gram (+), Gram negative (-) bacteria and *C. albicans*.

CONCLUSION

Further studies are being conducted to determine the characterization and identification of active components responsible for the antioxidant and antimicrobial activities. Natural products are commonly a source for active compounds that have important potential for developing new therapeutic agents. *Ononis* species can be introduced as new plant source for antioxidant and antimicrobial agents.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):130-135
DOI: 10.4274/tjps.14622

ORIGINAL ARTICLE

Phytochemical Analysis and Antidepressant Activity of *Ixora coccinea* Extracts in Experimental Models of Depression in Mice

Ixora coccinea Ekstrelerinin Fitokimyasal Analizi ve Sıçanlarda Depresyon Deney Modeli Üzerindeki Antidepresan Aktivitesi

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ABSTRACT

Objectives: The present study aims to assess the antidepressant activity of *Ixora coccinea* extracts in mice and phytochemical analysis of the active extract by GC-MS.

Materials and Methods: After oral administration of extracts, tail suspension test (TST), force swim tests (FST), and open field tests (OFT) were performed to assess the antidepressant activity. GC-MS analysis of methanol extract of *I. coccinea* was performed to ascertain the chemical constituents in the bioactive extract.

Results: The methanol extract of *I. coccinea* at dose of 100 and 200 mg/kg body weight, p.o. significantly reduced the total duration of immobility in the TST as well as FST ($p<0.01$). *I. coccinea* extracts showed no significant changes in locomotor activity in OFT.

Conclusion: The methanol extract of I. *coccinea* possesses antidepressant-like properties in mice with no significant effect on locomotor activity in OFT.

Key words: Antidepressant activity, forced swim test, GC-MS, *Ixora coccinea*, open field test, tail suspension test

ÖZ

Amaç: Bu çalışmada, *Ixora coccinea* ekstrelerinin sıçanlardaki antidepresan etkisi araştırılmış ve GC-MS tekniği kullanılarak aktif ekstrenin fitokimyasal analizi yapılmıştır.

Gereç ve Yöntemler: Ekstrelerin oral yolla uygulanmasının ardından, antidepresan etkinin değerlendirilmesi için kuyruktan asma testi (KAT), zorunlu yüzme testi (ZYT) ve açık alan testi (AAT) kullanılmıştır. Biyoaktif ekstredeki fitokimyasal bileşenleri aydınlatmak amacıyla *I. coccinea* metanollü ekstresi üzerinde GC-MS analizi yapılmıştır.

Bulgular: *I. coccinea* metanollü ekstresi 100 ve 200 mg/kg dozda, oral yolla uygulandığında, KAT ve ZYT testlerinde toplam hareketsizlik süresini anlamlı bir şekilde azaltmıştır ($p<0.01$). *I. coccinea* ekstreleri AAT'de lokomotor aktivite üzerinde anlamlı derecede bir etki göstermemiştir.

Sonuç: *I. coccinea* metanollü ekstresi sıçanlarda, AAT'de anlamlı derecede lokomotor aktiviteye neden olmadan antidepresan-benzeri etkiye sahiptir.

Anahtar kelimeler: Antidepresan aktivite, zorunlu yüzme testi, GC-MS, *Ixora coccinea*, açık alan testi, kuyruktan asma testi

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Received: 10.03.2017, **Accepted:** 13.07.2017

INTRODUCTION

Depression is a common illness. It was estimated that 350 million people are affected by this illness. Suicides can be the result of depression. It has been estimated that every year, approximately 1 million deaths occur due to depression. Depression is a heterogeneous disorder that often manifests with various symptoms at the psychological, behavioural, and physiologic levels.[1] Although treatment with commercially available antidepressant drugs is effective, a significant number of patients do not achieve continuous remission, despite intensive management, and only 60% of patients are responsive to currently available antidepressants.[2] The most common adverse effects of these antidepressants include agitation, nausea, headache, sleeplessness or drowsiness, and sexual problems. The impulsive clinical response to antidepressant drugs and high susceptibility to adverse effects are major clinical problems[3] thus, novel therapeutic agents are still needed to treat depression. Herbal treatment is another effective alternative to treat depression. The search for novel therapeutic plants that mitigate depressive disorders has been extensively explored over the past decade.[4] Thus, developing an effective and safe chemical compound that originates from traditional medicinal herbal remedies may provide a method to minimize adverse effects and to shorten the entire process and reduce the cost of drug discovery compared with conventional chemistry-based drug discovery.[5]

Ixora coccinea Linn. *(Rubiaceae)* is a bushy, rounded shrub found in the subtropical region of Florida. The plant is grown as ornamental plant in India. It is commonly known as Rangon (Bengali), flame of wood (English), Bandhaka (Sanscrit). The flowers contain cycloartenol esters[6] and have cytotoxic, hepatoprotective,[7] antitumor, antimicrobial activity,[8] and wound healing activity.[9] The leaves contain triterpene ixorene,[10] ixorapeptide I, ixorapeptide II,[11] and quercitrin,[12] and have cardioprotective,[13] antinociceptive,[14] antioxidant,[15] antidiarrhoeal,[16] antiasthmatic,[17] hypoglycaemic, and hypolipidemic activity,[18] and the roots show antioxidant activity.[19]

From a literature review, it appears that *I. coccinea* was used in folk medicine to treat various ailments such as in inflammatory conditions including sprains, eczema, contusions, and boils. The aim of the present study was to evaluate the antidepressant activity of *I. coccinea* stem extracts and perform gas chromatography-mass spectrometry (GC-MS) analysis of the active extract of *I. coccinea*.

MATERIALS AND METHODS

Harvesting and authentication of plant material

The *I. coccinea* stems were collected from the Dhule District, M.S., India, identified by Dr. S.G. Kotwal, HOD, Dept. of Botany, K.T.H.M. College, Nashik authenticated by Dr. Rao P.S.N., Scientist, B.S.I., Pune. The herbarium of the plant specimen has been deposited at B.S.I. Pune, the voucher specimen No. ARS-1 reference no: BSI/WC/Tech/2006/667.

Chemicals and drugs

Chloroform and methanol were obtained from Merck Ltd. (Mumbai, India). Gum acacia was from Sd fine-chem, (Mumbai, India). All chemicals and solvents used in the study were of analytical grade. Normal saline solution, imipramine, and fluoxetine were purchased from pharmacy shop.

Extraction of plant material

The stems of *I. coccinea* were air dried in the shade avoiding exposure to direct sunlight and were then pulverized in a grinder. The stem powder (#60-80) material was successively extracted using chloroform and methanol with the continuous extraction method with the help of Soxhlet apparatus. After completion of extraction, the solvent was distilled out and the extract was dried through vacuum drying.

The extract suspension was prepared in 1% acacia solution by trituration. The fluoxetine or imipramine tablet powder equivalent was suspended in normal saline solution. All solutions were freshly prepared whenever required.

Animals and treatment

The animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals, of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institutional Animal Ethical Committee of S.M.B.T. College of Pharmacy, Dhamangaon, Nasik, (M.S.), India (Registration No.1329/ac/10/CPCSEA). Male albino mice (22-26 g and 3 to 4 months) were used for the study. All animals were maintained under controlled conditions of temperature (22±2°C) and illumination (12 h light-dark cycle), with free access to food and water. Groups of six animals were structured and in order to reduce the influence of day variation all assays were conducted from 11 to 15 h and all assays were performed in a special noise-free room with controlled illumination.

The mice were divided into six groups (n=6) and received the following oral doses for 7 days:

Group I: Vehicle treated group - physiologic saline solution,

Group II: Test - Suspension of chloroform extract of *I. coccinea* in 1% acacia solution (100 mg/kg body weight per day),

Group III: Test - Suspension of chloroform extract of *I. coccinea* in 1% acacia solution (200 mg/kg body weight per day),

Group IV: Test - Suspension of methanol extract of *I. coccinea* in 1% acacia solution (100 mg/kg body weight per day),

Group V: Test - Suspension of methanol extract of *I. coccinea* in 1% acacia solution (200 mg/kg body weight per day),

Group VI: Positive control - fluoxetine or imipramine (10 mg/kg body weight per day).

Acute toxicity studies

The acute oral toxicity of the extracts of *I. coccinea* was tested using the up and down procedure as per the Organization for Economic Cooperation and Development test guidelines. Animals were dosed, one at a time, at 24 h intervals. Depending on the outcome, the dose for the next animals was adjusted up.

For further doses, a dose progression factor of 3.2 was used. The next dose was administered according to the mortality of the animal. The dose was increased if the animal survived. After reaching 2000 mg/kg body weight dose, four additional animals were administered the same dose.[20]

Assessment of antidepressant activity

Forced swim test (FST)

The FST was performed according to the method described by Porsolt et al.[21] with a minor modification. Mice were individually forced to swim in an open cylindrical container (diameter 14 cm, height 20 cm), with a depth of 15 cm of water at 25±2˚C. The experimental procedures were performed on days 4 and 7, 60 min after the administration of test components. Each mouse was judged to be immobile during 6 min. Immobility time in the FST was measured when the animals ceased struggling and remained motionless while floating in the water. The water in the containers was changed after each trial.[22]

Tail suspension test (TST)

The TST was performed according to the method described by Rosa et al.[23] Mice were suspended 50 cm above the table with the help of adhesive tape placed approximately 1 cm from the tip of the tail. The total duration of immobility during a 6-min period was scored manually. Immobility time in TST was measured when animals showed no limb or body movements, hung passively and completely motionless, except for movements caused by respiration.[24]

Open-field test (OFT)

The locomotor activity was assessed using an OFT according to the method described by Herrera-Ruiz et al.[25] in order to detect any link between locomotor activities and antidepressant activity of the *I. coccinea* extracts. The OFT was performed on mice that received treatments, which were used to determine immobility time in FST/TST 60 min before being observed in the open-field. Animals were placed individually in a box (30x30x15 cm), with the floor divided into 9 equal squares. After habituation to the arena for 5 min, the number of squares crossed with all paws, grooming, and rearing events were observed for 6 min. The box was cleaned with 10% ethanol after each trial.[26]

Phytochemical investigation of active extracts using GC-MS

The GC-MS analysis of the methanol extract of *I. coccinea* was performed at SAIF Panjab University Chandigarh, India. The chemical composition of the extracts was determined using a Thermo Scientific TSQ 8000 GC-MS with a direct capillary interface fused with silica capillary column TG 5MS (30 m x 0.25 mm, 0.25 µm). The methanol extract of *I. coccinea* were injected with helium used as a carrier gas at constant rate 1 mL/min, in pulsed splitless mode. The solvent delay was 2 min and the injection size was 1 µL. The mass spectrophotometric detector was operated in electron impact ionization mode with an ionizing energy of 70 eV and scanning from m/z 50-500. The GC temperature program started at 60˚C then elevated to 280˚C at a rate of 10˚C/min, with a 10 min hold at 280˚C. The injector, ion source, and detector temperatures were set at 250˚C, 230˚C, and 280˚C, respectively.[27,28] The peaks separated in GC-MS were identified using National Institute of Standards and Technology mass spectral databases.

Statistical analysis

All experimental results are given as the mean ± standard error of the mean. To compare the test and control groups, One-way analysis of variance (ANOVA), followed by Dunnett's test was performed. A value of $p<0.01$ was considered to be significant.

RESULTS

Extraction

Fresh 250 g of stems of *I. coccinea* yielded 8 g (3.20%) and 18.55 g (7.42%) of chloroform extract and methanol extract respectively.

Acute toxicity studies

Chloroform and methanol extract of *I. coccinea* showed neither behavioural changes nor mortality with an oral dose of 2000 mg/kg.

Antidepressant activity

The effects of I. coccinea extracts on the immobility time in the force swim test

The methanol extract of *I. coccinea* showed an antidepressant effect in the FST because it significantly reduced the immobility time compared with the vehicle treated group (184.00±4.76 sec.) (Figure 1). The immobility times of the methanol extract of *I. coccinea* and the chloroform extract of *I. coccinea* for doses of 100 and 200 mg/kg/day on the 7th day were found as 138.00±6.763 and 124.7±6.36 sec, and 172.70±6.259 and 160.00±7.849 sec, respectively. The chloroform extract of *I. coccinea* did not reduce immobility time significantly. The group treated with fluoxetine showed good activity (111.83±4.826 sec). No significant difference was observed in the immobility time of *I. coccinea* extracts on 4th day and the 7th day in the FST.

The effect of I. coccinea extracts on the immobility time in the TST

In the TST, the methanol extract of *I. coccinea* showed a significantly decreased immobility time compared with the vehicle-treated control group (180.00±6.23 sec) (Figure 2).

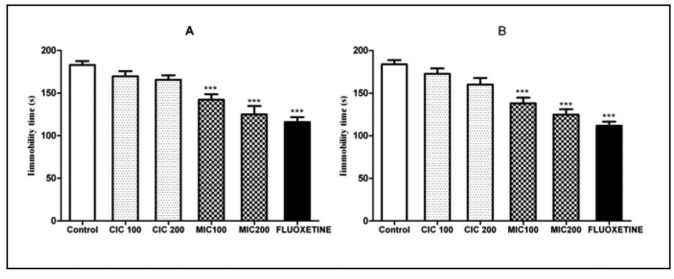

Figure 1. Effects of *I. coccinea* extracts (100 and 200 mg/kg) and fluoxetine (10 mg/kg) on the immobility time in the forced swim test on 4th day (A) and 7th day (B). The results are expressed as the mean ± standard error of the mean, n=6 in each group. ***p<0.001 VS the vehicle-treated control group. (CIC: Chloroform extract of I. coccinea, MIC: Methanol extract of *I. coccinea*)

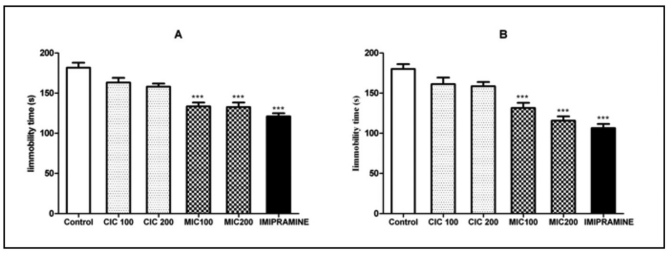

Figure 2. Effects of *I. coccinea* extracts (100 and 200 mg/kg) and imipramine (10 mg/kg) on immobility time in the tail suspension test on 4th day (A) and 7th day (B) in mice. The results are expressed as the mean ± standard error of the mean, n=6 in each group. ***p<0.001 VS the vehicle-treated control group. (CIC: Chloroform extract of I. coccinea, MIC: Methanol extract of *I. coccinea*)

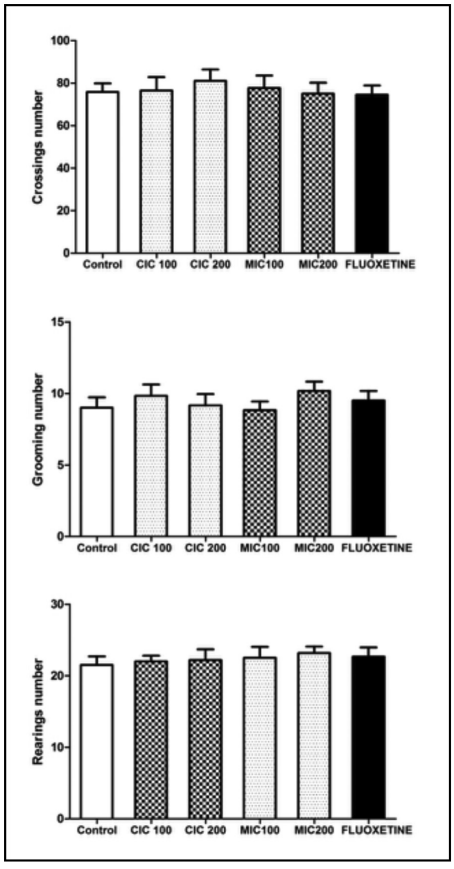

Figure 3. Effects of *I. coccinea* extracts (100 and 200 mg/kg) and fluoxetine (10 mg/kg) after 7th day administration on the number of crossings, and rearing and grooming activities in the open field test in mice. The results are expressed as the mean ± standard error of the mean, n=6 in each group. (CIC: Chloroform extract of *I. coccinea*, MIC: Methanol extract of *I. coccinea*)

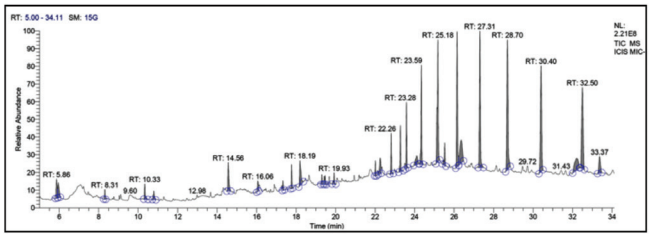

Figure 4. GC-MS chromatogram of methanol extract of *I. coccinea*

The mean immobility time of the methanol extract of *I. coccinea* treated group for 100 and 200 mg/kg dose was 131.50±6.515 and 115.8±5.78 sec, respectively. The chloroform extract of *I. coccinea* did not show a significant effect on immobility time (161.3± 8.044 and 158.50±5.476 sec). Imipramine, a non-selective reuptake inhibitor, which was used as positive control, significantly decreased the immobility time during the test session (106.50±5.156 sec). No significant difference was observed in the immobility time of *I. coccinea* extracts on the 4th and 7th days in the TST.

The effects of I. coccinea extracts in the open field test

No significant differences were observed in the number of squares crossed, and rearing and grooming activities between the vehicle-treated group and the *I. coccinea* extracts-treated group, as well as positive control group (Figure 3).

GC-MS analysis of pharmacologic active extract of I. coccinea

The results obtained from GC-MS analysis lead to the identification of the phytoconstituents present in the methanol extract of *I. coccinea*. The GC-MS spectra (Figure 4) indicated the presence of 2-Methoxy-4-vinylphenol, 3,4-Dimethoxy-6-methylpyrocatechol, 4-(3-hydroxy-1-propenyl)-2-methoxy-Phenol, methyl ester of Hexadecanoic acid, n-Hexadecanoic acid, methyl ester of 9-Octadecenoic acid (Z), Methyl stearidonate, Heneicosane, 16,17- Epoxyandrostane, Triacontane, Diisooctyl phthalate, Tetracosane, Stigmast-4-en-3-one, Squalene, and β-Sitosterol (Table 1).

DISCUSSION

Although *I. coccinea* has been used to treat nervous shock in traditional medicine, its specific neuropharmacologic activities have not yet been demonstrated. The FST and TST are the most common animal models used for screening antidepressant activity. In both tests, animals are placed in an inescapable situation and the decrease in immobility time indicates antidepressant-like activity.[29,30] In the FST, mice are forced to swim in a restricted space from which they cannot escape and it assumes a characteristic behaviour of immobility. This behavior reflects a state of despair or lowered mood, which can be reduced by agents that are therapeutically active in human depression. The TST also induces a state of immobility in animals similar to that in the FST. Fluoxetine is a classic selective serotonin reuptake inhibitor (SSRI), it is bound at the primary site of pre-synaptic serotonin transporter with very high affinity, and it has higher serotonergic activity than other classic SSRIs.[24] Imipramine prevents reuptake of noradrenaline and serotonin resulting in their increased availability in the synapse, and therefore, an increase in adrenergic and serotonergic neurotransmission.[31]

Psychostimulants, convulsants, and anticholinergics are able to increase locomotor activity in the OFT and give a false positive result in the TST and FST.[32] Agents that show a hyperkinesis effect also produce false positive effects in the TST and FST by reducing the immobility time.[33] Therefore, OFT was used to exclude these false effects that could be associated

Table 1. Compounds present in the methanol extract of *I. coccinea* using GC-MS analysis

Sr. no	Retention time	Name of compound	Mol. formula	Mol. weight
1.	10.80	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17
2.	14.56	3,4-Dimethoxy-6-methylpyrocatechol	$C_9H_{12}O_4$	184.18
3.	16.06	4-(3-hydroxy-1-propenyl)-2-methoxy- Phenol	$C_{10}H_{12}O_3$	180.20
4.	17.78	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45
5.	18.19	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42
6.	19.47	9-Octadecenoic acid (Z), methyl ester	$C_{19}H_{36}O_2$	296.48
7.	19.93	Methyl stearidonate	$C_{19}H_{30}O_2$	290.42
8.	22.02	Heneicosane	$C_{21}H_{44}$	296.57
9.	22.26	16,17- Epoxyandrostane	$C_{19}H_{30}O$	274.48
10.	22.82	Triacontane	$C_{30}H_{62}$	422.81
11.	23.28	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390.55
12.	23.59	Tetracosane	$C_{24}H_{50}$	338.65
13.	24.11	Stigmast-4-en-3-one	$C_{29}H_{48}O$	412.69
14.	25.53	Squalene	$C_{30}H_{50}$	410.71
15.	33.37	β-Sitosterol	$C_{29}H_{50}O$	414.70

with psychostimulants, convulsants, and anticholinergics or hyperkinesis.[2] The main difference between antidepressants and psychostimulants is that antidepressants would not increase locomotor activity.[34] In addition, the finding suggested that the reduction of immobility time elicited by the methanol extract in the FST as well as in the TST was a specific result of its antidepressant mechanism. In the TST and FST, the methanol extract of *I. coccinea* decreased the immobility time, which was not due to any psychostimulant, anticholinergic or convulsant effect, or hyperkinesis activity.

The methanol extract of *I. coccinea* decreased immobility time, whereas the chloroform extract showed no effect in either the TST or the FST. The immobility in TST and FST, referred to as behavioral despair in animals, is believed to reproduce a condition similar to human depression.[35]

In the present study, the methanol extract of *I. coccinea* was analyzed using GC-MS. To date, no reports exists on the GC-MS analysis of *I. coccinea* stems. From GC-MS analyses, bioactive extracts that show significant antidepressant activity contain fatty acid or esters such as methyl ester of hexadecanoic acid, n-hexadecanoic acid, methyl ester of 9-Octadecenoic acid (Z), Methyl stearidonate, Heneicosane, 16,17-Epoxyandrostane, Triacontane, Diisooctyl phthalate, Tetracosane; steroidals such as Stigmast-4-en-3-one, Squalene and β-Sitosterol; and phenolics such as 2-Methoxy-4-vinylphenol, 3,4-Dimethoxy-6-methylpyrocatechol, 4-(3-hydroxy-1-propenyl)-2-methoxy-Phenol. Phenolic compounds show good antidepressant activity;[36,37] the methanol extract of *I. coccinea* showed prominent antidepressant activity due to these phytoconstituents.

CONCLUSION

The present study provides the first evidence that the methanol extract of *I. coccinea* has significant antidepressant activity in the TST and FST models of depression in mice. The antidepressant activity may due to the presence of phenolic components. Further research is required to elucidate the mechanism of its action.

ACKNOWLEDGEMENTS

Principal, S.M.B.T. College of Pharmacy, Dhamangaon, India for providing necessary facilities and SAIF Panjab University, Chandigarh, India for providing GC-MS analysis facility.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):136-141
DOI: 10.4274/tjps.53825

ORIGINAL ARTICLE

Development of Andrographolide-Carboxymethyl Chitosan Nanoparticles: Characterization, *in vitro* Release and *in vivo* Antimalarial Activity Study

Andrografolid Karboksimetil Kitosan Nanopartiküllerinin Geliştirilmesi: Karakterizasyon, *in vitro* Salım ve *in vivo* Antimalaryal Aktivite Çalışması

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ABSTRACT

Objectives: The purpose of this study was to investigate the effect of andrographolide-carboxymethyl chitosan nanoparticles formation on the physical characteristics, *in vitro* release profile and *in vivo* antimalarial activity of andrographolide.

Materials and Methods: Nanoparticles were prepared by ionic gelation method-spray drying using $CaCl_2$ as the crosslinker with a composition of drug: polymer: $CaCl_2$=40: 250: 100. The obtained particles were evaluated for its size and morphology; physical state, drug content, *in vitro* drug release and *in vivo* antimalarial activity on *Plasmodium berghei* infected mice.

Results: The results of DTA and XRD showed that nanoparticle systems had a lower melting point and lower crystallinity degree. The drug dissolved from the nanoparticles was increased up to 6.5 times and the *in vivo* antimalarial activity was 1.65 times higher compared to andrographolide.

Conclusion: The formation andrographolide-carboxymethyl chitosan nanoparticles affected the physical characteristics of andrographolide. The decrease crystallinity of andrographolide resulted in a lower melting point of andrographolide. Such changes provided a positive impact to the drug dissolution and then its activity.

Key words: Andrographolide, carboxymethyl chitosan, *in vitro* release, *in vivo* antimalarial, ionic gelation, spray drying

ÖZ

Amaç: Bu çalışmanın amacı, andrografolid-karboksimetil kitosan nanopartiküllerinin oluşumunun andrografolidin fiziksel özellikleri, *in vitro* salım profili ve *in vivo* antimalaryal aktivitesi üzerine etkisini araştırmaktır.

Gereç ve Yöntemler: Nanopartiküller iyonik jelasyon yöntemi-püskürterek kurutma ile çapraz bağlayıcı olarak $CaCl_2$ kullanılarak etken madde: polimer: $CaCl_2$=40: 250: 100 bileşimi ile hazırlandı. Elde edilen partiküller büyüklükleri ve morfolojisi, fiziksel durumu, etken madde içeriği, *in vitro* etken madde salımı ve *Plasmodium berghei* ile enfekte sıçanlarda *in vivo* antimalaryal aktivite açısından değerlendirildi.

Bulgular: DTA ve XRD sonuçları, nanopartikül sistemlerinin daha düşük bir erime noktasına ve daha düşük kristallik derecesine sahip olduğunu gösterdi. Nanopartiküllerden çözünen etken madde 6.5 kat artmıştır ve *in vivo* antimalaryal etkinlik andrografolid ile karşılaştırıldığında 1.65 kat daha fazladır.

Sonuç: Andrografolid-karboksimetil kitosan nanopartiküllerinin oluşumu andrografolidin fiziksel özelliklerini etkiledi. Andrografolidin kristalinitesinin azalması, andrografolidin erime noktasının daha düşük olmasına neden olmuştur. Bu değişiklikler, etken maddenin çözünmesine ve daha sonra etkinliğine olumlu bir etki sağlamıştır.

Anahtar kelimeler: Andrografolid, karboksimetil kitosan, *in vitro* salım, *in vivo* antimalaryal, iyonik jelleşme, püskürterek kurutma

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Received: 04.02.2017, **Accepted:** 01.06.2017

INTRODUCTION

According to the World Health Organization report 2014, 17.1% of all essential medicines are classified as BCS II (high permeability and low solubility) and 10.6% of them are classified as BCS IV (low permeability and low solubility). A drug can be absorbed by the body and provide a pharmacologic effect for the body if the drug in a dissolved state.[1] Thus, a low solubility drug results in low bioavailability. Andrographolide, a diterpene lactone obtained from *Andrographis paniculata*, has extensive pharmacologic effects such as anti-inflammatory, anti-diarrhea, anti-HIV, anti-malarial, hepatoprotective, anticancer, antioxidant, and antihyperglycemic properties. Andrographolide application is restricted due to its low water solubility, short half-life time (2 hours) and low permeability. Pharmacokinetic studies showed that andrographolide was quickly absorbed and metabolized in rats and humans.[2,3] Several methods have been used to improve the low solubility of andrographolide. These methods are chemical modification, solid dispersion, liposomes, and nanoparticles. By improving the solubility, it is expected to increase the release rate and bioavailability.[2,4] Formation of nanoparticles andrographolide-eudragit® EPO increased bioavailability 2.2 times compared with pure andrographolide in oral administration.[5] Nanoparticles are a dispersion of solid particles with a diameter ranging between 10-1000 nm consisting of drug which is dispersed, trapped or enveloped in a matrix of nanoparticles. Nanoparticles can be used as a drug carrier in drug therapy or vaccine adjuvant, and because of their small size, they can increase the absorption of the drug into the biologic membrane by facilitated diffusion.[6-8]

Carboxymethyl chitosan is a derivative of the water-soluble chitosan. Having amine and carboxyl groups in the molecule enables it to be used as a carrier in drug delivery systems because these materials are biocompatible, biodegradable, and non-toxic.[9] Carboxymethyl chitosan nanoparticles can be prepared by emulsification, gelation ionic, coacervation, spray drying, sonication, emulsion droplet-coalescence, reverse micellar, and sieving. The mechanism of formation of carboxymethyl chitosan nanoparticles with an ionic gelation method is based on the electrostatic interaction between carboxyl groups of carboxymethyl chitosan and a positive charge of the crosslinker, $CaCl_2$. The addition of $CaCl_2$ is intended to form a bond between the divalent cations Ca^{2+} ions of $CaCl_2$ with -COO- of carboxymethyl chitosan.[9,10]

Malaria is an infectious disease caused by the parasite Plasmodium, which is characterized by fever, anemia, and splenomegaly.[11] There were 198 million cases of malaria and 584 thousand deaths (78% children under 5 years of age) due to malaria worldwide by 2013. Resistance to antimalarial drugs such as chloroquine and sulfadoxine-pyrimethamine is a problem that results in increased morbidity and mortality.[12] Therefore, the development of antimalarial drug delivery systems still needs to be achieved. In the present study, carboxymethyl chitosan nanoparticles of andrographolide were made by ionic gelation–spray drying to improve the physical properties and antimalarial activity of andrographolide. The particles obtained were evaluated for their morphology, physical state, *in vitro* release, and *in vivo* antimalarial activity on *Plasmodium berghei*-infected mice.

EXPERIMENTAL

Material

Andrographolide (RD Health Ingredients Co., Ltd.); Carboxymethyl chitosan (degree of substitution 81.9%, 96.5% degree of deacetylation, viscosity 1% 22 mPa.s, China Eastar Group Co., Ltd.); Calcium chloride $CaCl_2.2H_2O$ pro analysis (Merck); methanol; 96% ethanol pro analysis; distilled water; *Plasmodium berghei* ANKA strain obtained from the Eijkman Institute for Molecular Biology, Jakarta and maintained SATREPS ITD (Institute of Tropical Disease); Alceivers medium; Giemsa dye in phosphate buffer; absolute methanol.

Preparation of andrographolide-carboxymethyl chitosan nanoparticles

The andrographolide-carboxymethyl chitosan nanoparticles were prepared by ionic gelation using $CaCl_2$ as a crosslinker then spray dried. Two hundred fifty milligrams of carboxymethyl chitosan were dissolved in 100 mL of distilled water. Carboxymethyl chitosan solution was then poured into andrographolide solution (40 mg in 5 mL methanol) and stirred briefly at 500 rpm. The andrographolide-carboxymethyl chitosan solution was added into $CaCl_2$ solution (100 mg in 40 mL ethanol-water=1:9) and the mixture solution was stirred constantly for 4 hours at 500 rpm. Non-crosslinked particles were also prepared through simple mixing of the polymer solution and andrographolide solution. Dry particles were obtained by spray drying using SD Basic, LabPlant with 1.0 mm nozzle diameter at inlet temperature 100°C, flow rate 5 mL/min and pressure 2 mBar.

Particle morphology and size evaluation

The size, shape, and surface morphology of the particles were observed using scanning electron microscopy. Particles were embedded in a holder made of aluminum and coated with gold palladium prior to analysis. Pictures were taken at various magnifications at 20.00 kV.

Fourier transform infrared (FT-IR)

Particles were made as a pellet by mixing with KBr powder then pressed with a hydraulic pump to form a transparent pellet. Sample observation was conducted at wavelength 4000-450 cm^{-1} (Jasco FT-IR 5300, Easton MD, USA).

Differential thermal analysis (DTA)

Thermal analysis of the sample was conducted using a differential thermal analyzer (DTA FP-65 P-900 Thermal, Mettler Toledo, USA). About 5 mg of particles were placed in a crucible pan, sealed and observed for a thermogram. The thermogram was recorded at temperatures of 50 to 250°C with a heating rate 10°C/min.

X-ray diffractometry

X-ray diffraction analysis was conducted using a Phillips X'Pert X-ray diffractometer to evaluate the samples' crystallinity.

The light source employed was a Kα Cu Ni. The voltage and the current were set at 40 kV and 40 mA. Samples were analyzed at 2Ø and angle between 5-40º. The diffractogram of the andrographolide-carboxymethyl chitosan particles was compared with the diffractogram of base andrographolide.

Drug content

The andrographolide content in nanoparticle was determined using high-performance liquid chromatography (HPLC) Agilent 1100 Series using reverse phase with a mobile phase consisting of methanol-orthophosphoric acid (50:50, pH 3) at a wavelength of 228 nm. Five milligrams of accurately weighed particles were dissolved in 10.0 mL of methanol, filtered through a 0.2-µm membrane filter, and then the 5 mL sample was injected into the HPLC column. The assay is performed in triplicate.

The drug content was calculated using the equation below:

$$\% \text{ Drug content} = \frac{\text{drug amount}}{\text{particle weight}} \times 100\%$$

In vitro release study

The *in vitro* release study was performed in 50 mL 0.1% w/v sodium lauryl sulfate (SLS) media at 37±0.5˚C, 120 rpm using a water bath shaker. SLS was used to improve the dissolution of andrographolide.[13] Andrographolide-carboxymethyl chitosan particles equivalent to 2 mg of andrographolide were weighed accurately and put into the media. A 1.0-mL sample was taken at a predetermined time for 3 hours and the same volume of media was added after sampling. Andrographolide was also evaluated as control. The samples were analyzed using HPLC as described above. This evaluation was performed triplicate.

In vivo antimalarial activity test

The *in vivo* antimalarial activity test was conducted according to the method of Peter. The 4-day suppressive test of blood schizonticidal action.[14] Male mice aged 1.5-2 months of the strain Balb/C weighing 20-30 grams were supplied by the Department of Parasitology, Faculty of Medicine, University of Brawijaya, Malang. All mice were acclimatized in the animal house and were fed with standard diet and water *ad libitum*. The use of animals in this study was approved by the Animal Care and Use Committee (ACUC) of the Veterinary Faculty, Airlangga University (591-KE). All mice were injected intraperitoneally with 200 mL *Plasmodium berghei* infected mice's blood, which contained approximately 10^5 parasitized erythrocytes. Sixteen mice were used and were divided into four groups. Group 1 and group 2 were orally treated twice daily for four days with andrographolide suspended in carboxymethyl chitosan solution, and andrographolide-carboxymethyl chitosan particles suspended in water, respectively, with doses equivalent to 12.5 mg andrographolide/kg. Group 3 received carboxymethyl chitosan solution and group 4, as the untreated/control group, received water. Over a period of 6 days, tail blood was withdrawn and parasitemia was monitored by examining Giemsa-stained thin blood smears using an optical microscope. Parasitemia was calculated using the following equation:

$$\% \text{ parasitemia} = \frac{\text{number of parasitized erythrocytes}}{\text{number of erythrocytes}} \times 100\%$$

Percentage of parasitemia inhibition was calculated on day 5 using equation below.

$$\% \text{ inhibition} = 100\% - \frac{\text{\% parasitemia growth of treated group}}{\text{\% parasitemia growth of untreated group}} \times 100\%$$

RESULT AND DISCUSSION

Figure 1 shows the morphology of andrographolide-carboxymethyl chitosan nanoparticles and non-cross-linked andrographolide-carboxymethyl chitosan. Cross-linked andrographolide-carboxymethyl chitosan was seen as non-spherical with a hollow shape in a micrograph, whereas the non-cross-linked andrographolide-carboxymethyl chitosan produced more spherical and smooth particles. The andrographolide crystal was observed in cross-linked particles. It indicated that andrographolide was solidified thus hindered the formation of spherical and smooth particles shape. Cross-linked andrographolide-carboxymethyl chitosan nanoparticles had a range of sizes from 600 nm to 3000 nm, whereas the non-cross-linked andrographolide-carboxymethyl chitosan particle sizes were within the range of 500 nm to 2500 nm.

DTA

From the DTA thermogram in Figure 2, it was identified that andrographolide had a melting range 231.6˚C with a sharp

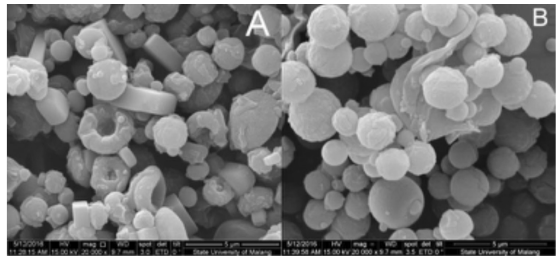

Figure 1. SSEM of crosslinked andrographolide-chitosan particles (A) and non crosslinked andrographolide- chitosan particles (B) (magnification 20000x)

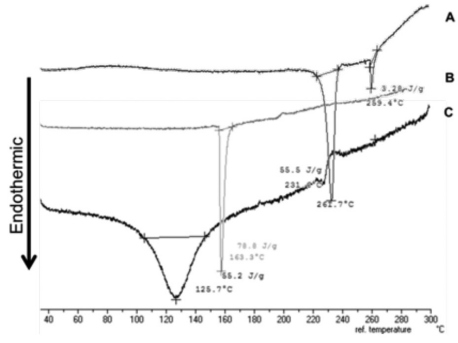

Figure 2. DTA thermogram of andrographolide (A), and non-crosslinked andrographolide-chitosan particles (B), crosslinked andrographolide-chitosan particles (C)

endothermic peak, and non-cross-linked andrographolide-carboxymethyl chitosan particles had a sharp endothermic peak with melting point of 162.9˚C. The thermogram of andrographolide-carboxymethyl chitosan nanoparticles shifted to the lower melting point of 125.7˚C with a wider endothermic peak compared with andrographolide and the non-cross-linked andrographolide-carboxymethyl chitosan particles. It indicated that the formation of cross-linked particles resulted in lower ordered structural molecule patterns compared with the non-cross-linked particles and base andrographolide.

X-ray diffractrometry

As shown in Figure 3, the diffractogram of andrographolide indicated crystalline peaks with high intensity on 2Ø 9.83˚, 14.81˚, 15.69˚, 15.85˚. $CaCl_2$ itself had a diffraction peak at 14.74˚ 2Ø. In the diffractogram of andrographolide-carboxymethyl chitosan physical mixture, several crystalline peaks with low intensity at 12.14˚, 15.77˚ and 18.55˚ were detected.

The diffractogram of andrographolide-carboxymethyl chitosan nanoparticles showed that diffraction peaks of andrographolide, carboxymethyl chitosan, and $CaCl_2$ disappeared, but new crystalline peaks at 31.63˚ 2Ø appeared. These results showed that the formation of cross-linked particles of andrographolide-carboxymethyl chitosan prepared by ionic gelation-spray drying had lower crystallinity compared with andrographolide itself.

Drug content

From the drug content evaluation using HPLC, the andrographolide content in the particles was found as 12.09±0.26%. The result was further used to calculate the amount of andrographolide-carboxymethyl chitosan for the *in vitro* release test and *in vivo* antimalarial activity test.

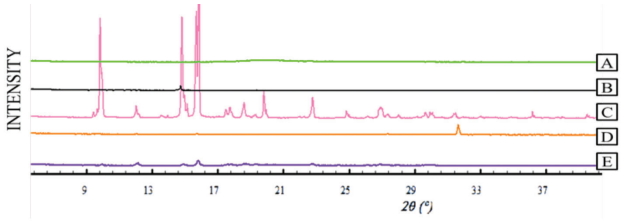

Figure 3. X-ray diffractogram of (A) carboxymethyl chitosan, (B) $CaCl_2$, (C) andrographolide (D) crosslinked andrographolide-chitosan particles, dan (E) physical mixture of andrographolide-chitosan

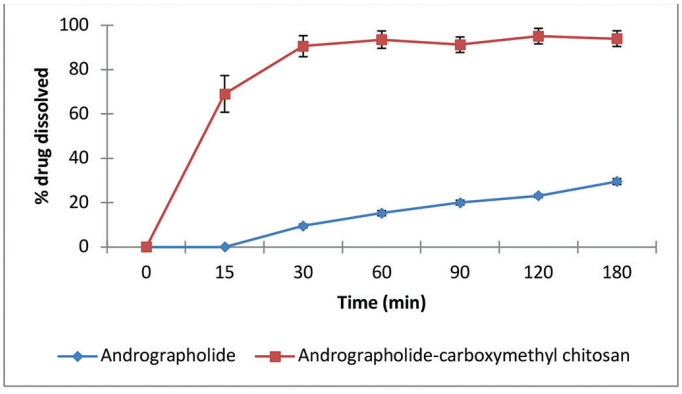

Figure 4. *In vitro* release profile of andrographolide-carboxymethyl chitosan particles and andrographolide in 0.1% SLS media at 37±0.5˚C (n=3)

In vitro release study

The *in vitro* release test was performed in 0.1% SLS to facilitate drug dissolution. The result demonstrated that the amount of andrographolide dissolved in 15 min from the nanoparticle systems (69.06%) was greater compared with andrographolide. After 30 min, the amount of andrographolide dissolved from the nanoparticles was up to 10 times higher than with base andrographolide (Figure 4).

The slope that indicated the release rate of andrographolide from carboxymethyl particles and base andrographolide were 12.7158±0.8054% dissolved/$min^{1/2}$ and 2.0221±0.2702% dissolved/$min^{1/2}$, respectively. The result indicated that the formation of andrographolide-carboxymethyl chitosan could increase the release rate of andrographolide 6.3 times compared with andrographolide itself. This was due to the formation of particles prepared by ionic gelation-spray drying lead to changes in the crystallinity of the drug as well as has being shown by the results of the DTA thermogram and X-ray diffractogram in Figure 2 and Figure 3. The entrapment of andrographolide into the cross-linked carboxymethyl chitosan continued with fast solidification of the particles during the spray drying process, which caused inhibition of crystal growth, could result in an amorphous form and crystal size reduction of andrographolide. The changes of crystallinity structure into the amorphous form and reduction of particle size of poorly soluble drug would be advantageous because it would enhance the solubility and then its bioavailability.[15,16]

In vivo antimalarial activity test

Figure 5 shows that parasitemia growth occurred during the evaluation in all groups. The treated groups led to a slow growth of parasitemia because the untreated/control group

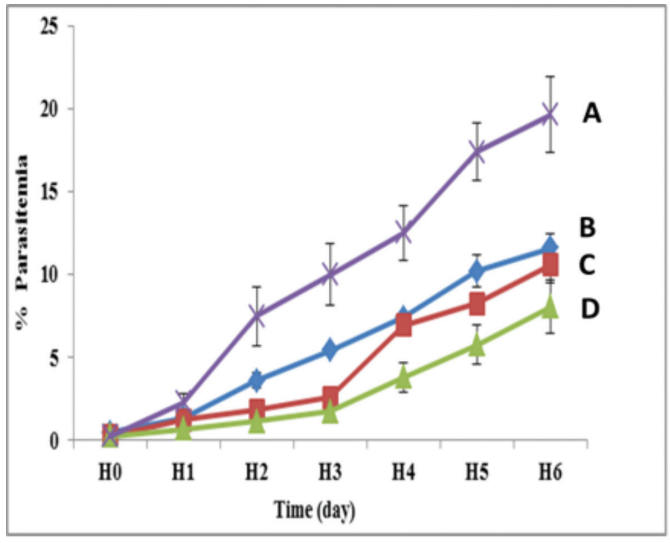

Figure 5. Parasitaemia growth curve of *Plasmodium berghei* infected mice of (A) control group, (B) andrographolide treated group, (C) carboxymethyl chitosan treated group and (D) andrographolide-carboxymethyl chitosan particles treated group

had rapid growth parasitemia. The antimalarial activity test results presented in Figure 5 and Figure 6 revealed that the increasing number of *Plasmodium berghei* infected erythrocytes (parasitaemia) were lower than the control/untreated group in all treated groups.

The growth inhibition of parasitemia on day five of andrographolide system-carboxymethyl chitosan nanoparticles-treated group was 71.27±6.83%, which was higher compared with the andrographolide-treated group (43.30±1.83%) and carboxymethyl chitosan-treated group (45.48±3.71%). The formation of andrographolide nanoparticles with carboxymethyl chitosan changed the physical state of andrographolide, which lowered its melting point and degree of crystallinity, as shown in Figure 2 and Figure 3. These results will improve the dissolution of andrographolide and further, will provide a favorable effect on its activity. The parasitemia growth inhibition of the andrographolide-carboxymethyl chitosan particles increased 1.65 times compared with andrographolide and was statistically significantly different (Table 1).

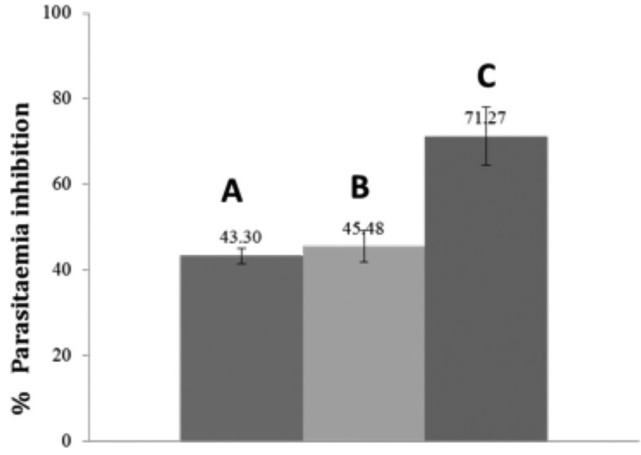

Figure 6. Histogram of parasitaemia inhibition percentage of *Plasmodium berghei* infected mice of (A) andrographolide treated group, (B) carboxymethyl chitosan treated group and (C) andrographolide-carboxymethyl chitosan particles treated group

Table 1. One-way ANOVA (p=0.05) to determine the effect of andrographolide, carboxymethyl chitosan and andrographolide-carboxymethyl chitosan particles on parasitaemia inhibition of *0* infected mice

Group	n	% Parasitaemia inhibition ± SD	ANOVA	
			Result	Conclusion
Andrographolide treated group	4	43.30±1.83[a]	F=11.373 p=0.002	Significantly different
Carboxymethyl chitosan treated group	4	45.48±3.71[a]		
Andrographolide-carboxymethyl chitosan particles treated group	4	71.27±6.83[b]		

Note: [a,b] Signs refer to no difference between the groups. n: Sample number

CONCLUSION

The formation system of andrographolide-carboxymethyl chitosan nanoparticles affected the physical characteristics of andrographolide. The crystallinity decrease of andrographolide resulted in a lower melting point of andrographolide. Such changes had a positive impact on the drug dissolution and then its activity. The release rate of andrographolide from carboxymethyl chitosan nanoparticles increased up to 6.3 times and *in vivo* antimalarial activity in *Plasmodium berghei*-infected mice was significantly enhanced up to 1.65 times compared with base andrographolide.

ACKNOWLEDGEMENTS

This research was financially supported by Research Grant of Faculty of Pharmacy, Airlangga University, 2016.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):142-148
DOI: 10.4274/tjps.54265

ORIGINAL ARTICLE

Development and *in vitro* Evaluation of Voriconazole Nanoparticle Formulation for Mucosal Application

Mukozal Uygulama için Vorikonazol Nanopartikül Formülasyonunun Geliştirilmesi ve *in vitro* Değerlendirilmesi

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ABSTRACT

Objectives: This study aimed to prepare and evaluate mucoadhesive nanoparticle formulations of voriconazole, an antifungal drug, for mucosal application. It was also aimed to develop and validate a HPLC method of voriconazole.

Materials and Methods: In this study, mucoadhesive nanoparticles containing voriconazole were prepared using a coating polymer of chitosan. The obtained nanoparticles were characterized via particle size, polydispersity index, zeta potential measurement, and mucoadhesion studies. Drug loading capacity was tested for determination of the voriconazole amount in the nanoparticles. *In vitro* drug release was also examined. The HPLC method was validated for linearity, accuracy, precision (repeatability and reproducibility), specificity, stability, limits of detection (LOD), and limit of quantification (LOQ).

Results: *In vitro* characterization results of the mucoadhesive nanoparticle formulation containing voriconazole was found to be appropriate with a small particle size of 217.1±4.2 nm, a narrow polydispersity index of 0.335±0.042, 99.052±0.424% drug loading, and a positive zeta potential value of +26.82±0.4 mV. According to a mucoadhesive study, it can be concluded that the nanoparticle was able to interact with mucin due to ionic interaction. Also, the turbidity of nanoparticle/mucin dispersion was higher than the turbidity of mucin dispersion itself. Based on the *in vitro* drug release, no burst effect was observed, indicating that voriconazole was homogeneously dispersed in the nanoparticle dispersion and that no significant amount of drug was adsorbed onto the nanoparticle surface. The release was found to follow a non-Fickian diffusion mechanism with first-order drug release. The proposed HPLC method was simple, highly sensitive with good linearity, accurate, precise, specific, and stable, showing that the method is useful for routine quality control.

Conclusion: This study has shown that the mucoadhesive nanoparticle formulation containing voriconazole reported here is a promising candidate for the local treatment of mucosal diseases. The developed HPLC method can be succesfully applied to pharmaceutical preparations containing voriconazole.

Key words: Voriconazole, mucoadhesive nanoparticle, chitosan, local application, HPLC

ÖZ

Amaç: Bu çalışma, mukozal uygulama için antifungal bir ilaç olan vorikonazolün mukoadezif nanopartikül formülasyonunu hazırlamayı ve değerlendirmeyi amaçlamıştır. Ayrıca vorikonazolün HPLC yöntemi geliştirilmiş ve valide edilmiştir.

Gereç ve Yöntemler: Bu çalışmada, kitozan kaplama polimeri kullanılarak vorikonazol içeren mukoadezif nanopartikül hazırlandı. Elde edilen nanopartikül formülasyonu, partikül boyutu, polidispersite indeksi, zeta potansiyeli ölçümü ve mukoadezyon çalışmaları ile karakterize edildi. İlaç yükleme kapasitesi nanopartikülde vorikonazol miktarının belirlenmesi için gerçekleştirildi. *İn vitro* ilaç salınımı da incelendi. HPLC yöntemi doğrusallık, doğruluk, kesinlik (tekrar edilebilirlik ve tekrar elde edilebilirlik), özgünlük, stabilite, LOD ve LOQ ile valide edildi.

Bulgular: Vorikonazol içeren mukoadezif nanopartikül formülasyonu, 217.1±4.2 nm küçük partikül boyutu, 0.335±0.042 dar polidispersite indeksi, %99.052±0.424 ilaç yükleme kapasitesi, +26.82±0.4 mV pozitif zeta potansiyel değeri ile uygun bulunmuştur. Mukoadezyon çalışmasına göre, nanopartikülün, iyonik etkileşim nedeniyle müsinle etkileşime girdiği sonucuna varılabilir. Ayrıca nanopartikül/müsin dispersiyonunun bulanıklığı, müsin dispersiyonunun bulanıklığından daha fazla bulunmuştur. *İn vitro* ilaç salım çalışmasına göre, vorikonazolün nanopartikül dispersiyonunda homojen olarak dağılmış olduğu ve nanopartikül yüzeyi üzerinde önemli miktarda ilacın adsorbe edildiğini gösteren ani salım etkisi gözlemlenmemiştir. İlaç salımı, birinci dereceden ilaç salımı ile Fick Kanunu'na uymadığını göstermiştir. Önerilen HPLC yöntemi, rutin kalite kontrolü için basit, çok hassas, doğrusal, kesin, hassas, özgün ve stabildir.

Sonuç: Bu çalışma, vorikonazol içeren mukoadezif nanopartikül formülasyonunun, mukozal hastalıkların lokal tedavisinde umut verici bir aday olduğunu göstermiştir. Geliştirilen HPLC yöntemi, vorikonazol içeren farmasötik preparatlar için başarılı bir şekilde uygulanabilir.

Anahtar kelimeler: Vorikonazol, mukoadezif nanopartikül, kitosan, lokal uygulama, HPLC

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Received: 12.04.2017, **Accepted:** 13.07.2017

INTRODUCTION

Voriconazole (VRZ) ((2*R*,3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butan-2-ol) is a novel broad spectrum triazole antifungal agent for the treatment of serious infections caused by *Aspergillus, Fusarium, Scedosporium*, and resistant *Candida* species.[1] Since its release by the United States Food and Drug Administration in May 2002, VRZ has established itself as the first-line treatment for invasive aspergillosis and proven useful in other fungal infections that are resistant and refractory to standard antifungal therapies.[2,3] In Europe, the drug has been approved by the European Medicines Agency (EMEA) since March 2002 for the treatment of invasive aspergillosis, fluconazole-resistant strains of *Candida* species, and serious infections caused by *Scedosporium* spp. and *Fusarium* spp. (EMEA 2002). VRZ is a synthetic derivative of fluconazole, one of the triazole moieties in fluconazole is replaced by a fluoropyrimidine group and an alpha methyl group is also introduced. It is more active than fluconazole and itraconazole against *Candida* species.[4,5] The drug has reported adverse effects of visual disturbances, hepatic toxicity, headache pain, and dermatologic reactions, as well as serious cytochrome P450-mediated drug interactions.[6,7] Because of the well-reported adverse effects, drug interaction, and the ominous risk of drug resistance of systemic VRZ, a local formulation without the aforementioned risks is needed in clinical practice.

Local drug delivery is frequently used for the treatment of localized disorders. The main advantages of local administration are the ability to deliver the active agent directly to the site and the maintenance of the required concentration of active substance at the site for a prolonged period.[8] A great deal of attention has been devoted to the development of mucoadhesive drug delivery systems. Mucoadhesive nanoparticle (NP) formulations have raised widespread interest for their recognised potential for improving the bioavailability of drugs and maintaining the local effect in the targeted area. The increased contact time and localization of the drug is due to applying nanoparticles (NPs) of VRZ, which are made mucoadhesive, thus enhancing its delivery. This approach prolongs the activity of the active substance as well as reduces the frequency of administration. A possible added advantage of mucoadhesive NP is that particulates have the advantage of being relatively small and are thus accepted by patients.

NPs coated with chitosan (CSH) have attracted a special interest for drug delivery through the mucosal routes because of their ability to interact with the negatively charged sites on the mucosa surface, prolonged retention time, mucoadhesive properties, and increased local concentration of NPs.[9,10] CSH, which is strong mucoadhesive, nontoxic, cationic, biocompatible, biodegradable, and has mucoadhesiveness as well as antibacterial, antifungal, and antitumor activity, is a suitable polymer for mucoadhesive drug formulations.[11-13] These functional properties provide suitability and extensive pharmaceutical applications; therefore, CSH-coated NPs have reached an important position in the arena of drug delivery.

The objective of this study was to prepare a mucoadhesive NP formulation of VRZ. CSH was used as coated polymer to provide the mucoadhesive property to the NP system. *In vitro* characterization of the prepared mucoadhesive NP formulation was performed. An additional aim was to develop and validate a simple, rapid, and economic high-performance liquid chromatography (HPLC) method for the analysis of VRZ as per International Council for Harmonisation of Technical Requirements (ICH) guidelines.

MATERIALS AND METHODS

Materials

VRZ were obtained from Sigma-Aldrich (St Louis, MO, USA). Eudragit (EUD) RS 100 as gifts from Karadeniz Chemical Company (Karadeniz, Turkey). High-molecular-weight CSH (Brookfield viscosity [1%, m/V, in 1% acetic acid solution at 25°C]; 800,000 mPa.s.75% deacetylated) and HPLC grade acetonitrile were purchased from Sigma-Aldrich. All other materials were of analytical grade.

HPLC system

HPLC was conducted using a Hewlett Packard series 1100-1200 HPLC apparatus (Santa Clara, CA, USA) equipped with an UV detector set at 255 nm using a C18 column (5 µm, 4.6×250 mm). The injection volume was 50 µL. The mobile phase, fluxed at 1.5 mL/min, was a mixture of acetonitril:water (35:65 v/v). The flow rate was maintained at 25±1°C.

Preparation of stock solutions and standard working solution

Stock solution of VRZ was prepared by dissolving 1 mg of drug in 10 mL methanol. VRZ concentrations in the working solution chosen for the calibration curves were 2.5, 5, 10, 15, 20 and 30 µg/mL. All samples were filtered through an aqueous 0.2- µm pore-size membrane filter before injection.

Validation of HPLC method

The developed HPLC method was validated according to ICH guidelines including the determination of linearity, calibration curve, accuracy, precision (repeatability and reproducibility), specificity, stability, limit of detection (LOD), and limit of quantification (LOQ) of analysis in spiked samples.[14-16]

Specificity

The specificity of an analytical method is its ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. These parameters were determined by comparing the chromatograms of the VRZ standard, methanol, and mobile phase.

Linearity

The linearity between the peak area and concentration was analyzed using a calibration curve obtained from standard solutions of VRZ (2.5 to 30 µg/mL). In addition, it was evaluated using linear regression analysis, which was calculated by the least-square regression analysis.

Accuracy and recovery

The accuracy study is the closeness of test results obtained by the method to the true value and is defined recovery. The prepared three standard solutions (10, 15, 20 µg/mL) were injected six times at different levels as a test sample.

Precision

The precision of the assay was determined with repeatability and reproducibility. Repeatibilty was calculated from six replicated injections of freshly prepared VRZ test solution in the same equipment at a concentration value of 10 µg/mL of the intended test concentration value on the same day. For reproducibility study, 10 µg/mL sample was prepared and injected into HPLC system as per the test procedure. The peak area of VRZ was determined and precision was reported as % RSD.

LOD and LOQ

LOD and LOQ tests for the procedure are performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyte that can be reproducibly quantitated above the baseline noise that gives S/N = 10.

Stability

For a short-term stability study, a sample solution of VRZ was prepared and analyzed initially and also at the end of 48 hours by keeping the solution at room temperature.

Preparation of mucoadhesive NP formulation

The NPs were prepared by adapting the spontaneous emulsification technique previously described by Bodmeier et al.[17] The final preparation method was established in accordance with Rençber et al.'s[18] study. In brief, 2.5% of EUD RS polymer and 0.05% VRZ were dissolved in 25 mL ethanol. The alcoholic solution mixture was added dropwise (3 mL/min) to 50 mL of 0.025% w/v aqueous chitosan solution under continuous magnetic stirring at 800 rpm. The formed CSH-coated NP was further stirred for 48 hours at room temperature.

Measurement of particle size (PS), polydispersity index (PI) and zeta potential (ZP)

The PS, PI and ZP were measured at 25°C using a Nano-ZS Zetasizer (Malvern Instruments, Malvern, UK). The PS and PI values were obtained by averaging ten measurements at an angle of 173° using disposable cells. The ZP was calculated from the electrophoretic mobility using the Helmholtz-Smoluchowski equation under an electrical field of 40 V/cm. The processing was performed using the software included within the system (n=5).

Mucoadhesive evaluation: ZP determination and turbidimetric measurement

Two *in vitro* methods were used to assess the mucoadhesive evaluation of NP.[19-21] In the first method, the mucoadhesive property of NP with VRZ was evaluated by measuring the changes of ZP on interaction with negatively charged mucin. The mucoadhesive NP containing VRZ was incubated at 37°C in 0.1% mucin dispersion. The ZP of the NP was measured over 6 hours. The alteration of ZP of the CSH-coated NP with VRZ indicates interaction with mucin.

Turbidimetric measurements of mucoadhesive NP containing VRZ was compared with mucin dispersion at 650 nm by ultraviolet–visible spectrophotometer. The accurately mucoadhesive NP (5 mL) was added to 5 mL aqueous mucin dispersion and stirred at 200 rpm. The turbidity of the dispersions was measured at certain time intervals over 6 hours and compared with the turbidity of the mucin dispersion. The increase in turbidity of mucin: mucoadhesive NP dispersion with VRZ indicated mucoadhesive property.

Drug loading (DL) capacity

The DL capacity of NP was determined by dissolving 0.04 mL of the NP in 8 mL methanol with vortex, followed by a validated HPLC assay for VRZ. The DL capacity was calculated according to the following equations:[18]

$$DL = \frac{\text{Total amount of VRZ-The amount of free VRZ}}{\text{Total amount of formulation components)}} \times 100$$

In vitro drug release studies

The release of VRZ from 4 mL of NP was assessed using a dialysis bag (cellulose membrane, 12,000-14,000-molecular-weight cutoff Spectrum Labs, Rancho Dominguez, CA, USA into phosphate buffer saline (PBS) at 37°C±0.5°C, and stirred continuously with a magnetic stirrer at 300 rpm for 24 hours. The amount of the drug in the receiving solution was analyzed using the validated HPLC method. Sink conditions were maintained in the receptor compartment during *in vitro* release studies (n=5).

Determination of drug release mechanism

The dissolution data were fit to the Peppas equation, and best-fit parameters were calculated to determine the release mechanism of the tablets.[22]

RESULTS

HPLC system

An HPLC method for quantitative analysis of VRZ in mucoadhesive NP formulation was developed and validated. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. There was no interference from the methanol and mobile phase at VRZ peaks. The chromatogram of the VRZ standard presented a peak in the retention time of 13.669 min; the total analysis time was 15 min (Figure 1).

A 6-point calibration curve was constructed covering a concentration range from 2.5 to 30 µg/mL for standard solution of bulk VRZ. The determination correlation coefficient (R^2) for a regression line is 0.9931 with the linear regression equation y=42.537x-46.906. The analyses of calibration are shown in Figure 2.

The HPLC area responses for accuracy determination were evaluated and the mean recovery data of VRZ were within the range of 89.702 and 105.500% (Table 1). The mean % relative

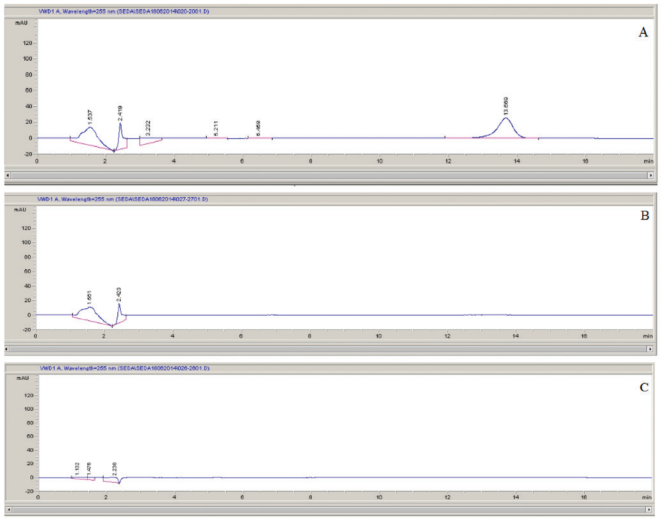

Figure 1. The chromatograms obtained of VRZ in standard solution (A), methanol (B) and mobile phase (C)

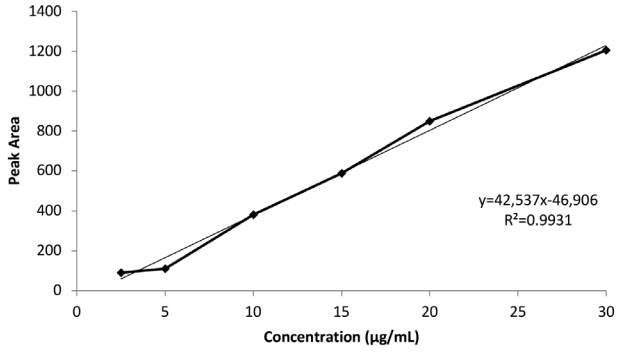

Figure 2. The regression line for VRZ

standard deviations (R.S.D.) were between 0.112 and 0.542%, thereby satisfying the acceptance criteria for the study (R.S.D. maximum 2.0%). Hence, the accuracy of the method was confirmed, this method can be used for further studies.

As Table 2 shows, the percentage of mean recovery values of precision study were found as 100.541%-100.616%, with R.S.D. ranges 0.028-0.392.

The determined values of LOD and LOQ were 0.804 µg/mL and 1.608 µg/mL, respectively.

During the storage of the solution at room temperature, the solutions were stable for 48 hours (Table 3).

Preparation and characterization of mucoadhesive NP formulation

In our study, mucoadhesive NP formulation was successfully prepared adapting the spontaneous emulsification technique in accordance with Rençber et al.'s[18] study. The PS, PI and ZP were found as 217.1±4.2 nm, 0.335±0.042, and 26.82±0.4 mV, respectively.

To obtain insight into the mechanism of mucoadhesive NP with VRZ: mucin interaction, ZP measurements of their dispersions were performed and the results are shown in Figure 3A. As seen in Figure 3B, the absorbance of 0.1% aqueous mucin

Table 1. Percent recovery and coefficient of variations

Concentration	Injection times of test solution	Amount found concentration (µg/mL)	Recovery %
10 µg/mL	1	10.111	101.113
	2	10.057	100.573
	3	10.050	100.502
	4	10.076	100.761
	5	9.994	99.938
	6	9.963	99.632
	Mean	10.042	100.420
	S.D.	0.054	0.544
	R.S.D. %	0.542	0.542
15 µg/mL	1	13.438	89.589
	2	13.472	89.816
	3	13.517	90.115
	4	13.443	89.617
	5	13.430	89.532
	6	13.432	89.546
	Mean	13.455	89.702
		0.034	0.227
	R.S.D. %	0.253	0.253
20 µg/mL	1	21.101	105.505
	2	21.069	105.344
	3	21.099	105.497
	4	21.135	105.673
	5	21.116	105.579
	6	21.081	105.403
	Mean	21.100	105.500
	S.D.	0.024	0.118
	R.S.D. %	0.112	0.112

S.D.: Standard deviation, R.S.D.: Relative standard deviations

dispersion at λ=650 nm was used as a reference for the turbidimetric study.

The DL capacity of the mucoadhesive NP formulation was found as 99.052±0.424%. The *in vitro* VRZ release from the NPs using a dialysis bag for 24 hours is shown in Figure 4.

The values of n, log k, and r^2 calculated using the Peppas equation were found as 0.7477, 0.1946, and 0.9962, respectively.

$$\text{Log}\left(\frac{M_t}{M_\infty}\right) = \log k + n.\log t$$

where M_t/M_∞ is the fractional release, k is the diffusional constant, and n is the diffusional exponent that characterizes the drug release mechanism.

Table 2. The results of precision study for 10 µg/mL bulk VRZ solution

	Repeatability		Reproducibility	
Injection times of test solution	Amount found concentration (µg/mL)	Recovery %	Amount found concentration (µg/mL)	Recovery %
1	10.111	101.113	10.057	100.573
2	10.057	100.573	10.050	100.502
3	10.050	100.502	10.053	100.526
4	10.076	100.761	10.057	100.573
5	9.994	99.938	10.055	100.549
6	10.081	100.808	10.053	100.526
Mean	10.062	100.616	10.054	100.541
S.D.	0.039	0.395	0.003	0.028
R.S.D.	0.392	0.392	0.028	0.028

S.D.: Standard deviation, R.S.D.: Relative standard deviations, VRZ: Voriconazole

Table 3. The results of stability study for 10 µg/mL bulk VRZ solution

Injection times of test solution	Amount found concentration (µg/mL)	Recovery %
1	10.057	100.573
2	10.048	100.479
3	10.041	100.408
4	10.041	100.408

S.D.: Standard deviation, R.S.D.: Relative standard deviations, VRZ: Voriconazole

DISCUSSION

HPLC methods have been widely employed in pharmaceutical analysis due to the ease of performance, specificity, sensitivity, and the analysis of samples of complex nature. In this study, HPLC was proposed to quantify VRZ in mucoadhesive NP formulation. Mobile phase selection was based on peak parameters, run time, ease of preparation, and cost. All samples were maintained at 25˚C in the autosampler prior to injection. A volume of 50 µL of each sample was directly injected into the HPLC system. Acceptable separations (Figure 1), with a retention time of 13.669 min for VRZ was obtained using a C-18 column and a mobile phase composed of acetonitril:water (35:65). The column temperature was maintained at 25˚C. Monitoring of VRZ was realised with UV detection at a wavelength of 255 nm.

To be considered specific, an analytical method should demonstrate that it can separate and quantify the drug from impurities, degradation products, and excipients. To evaluate the specificity of the analytical method, the VRZ test solution, methanol, and mobile phase were injected into the chromatographic system. There was no interference from the methanol and mobile phase at VRZ peaks.

The linearity is its ability to elicit test results that are directly or by a well-defined mathematical transformation, proportional

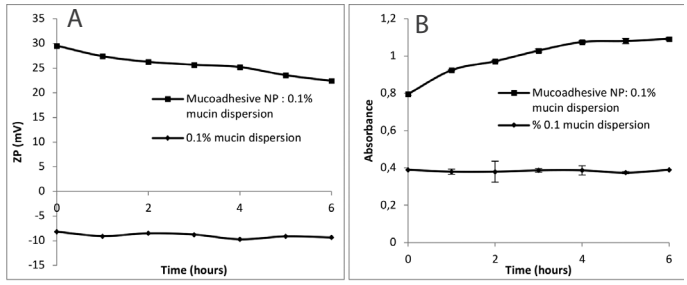

Figure 3. (A) Estimation of the ZP of the mucoadhesive NP during incubation in 0.1% aqueous mucin dispersion (B) estimation of the interaction between mucoadhesive NP and mucin dispersion by turbidimetric assay

ZP: Zeta potential, NP: Nanoparticle

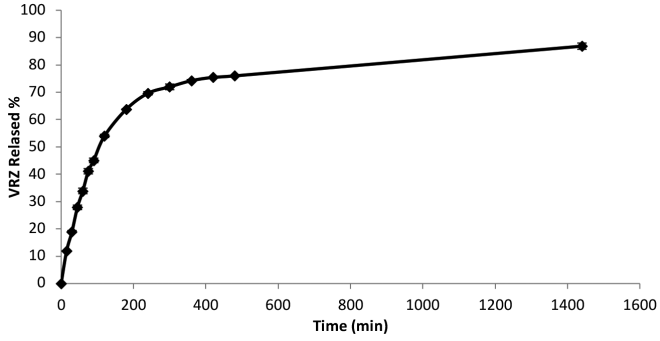

Figure 4. *In vitro* drug release of mucoadhesive NP

VRZ: Voriconazole

to analyte concentration in samples within a given range.[23,24] Linearity data indicate that the VRZ peak areas are linear over a concentration range of 2.5-30 µg/mL. A linear relationship between the peak area and concentration of VRZ was observed. A correlation coefficient was equal to 0.99, which indicated a strong linear relationship between the variables. The standard deviation of the slope and intercept were low.

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. It can be defined as the ratio of obtained concentration values to true values and its percentage of recovery.[25]

Precision studies for an analytical procedure are the degree of agreement among individual test results when an analytical method is used repeatedly for multiple samplings of a homogeneous sample under the same conditions. In terms of the method precision study of our experiment, 10 µg/mL solutions were injected six times into the system and the percentage of recovery was evaluated. Because the percentage of recovery has been found almost 100 and the R.S.D. value less than the acceptance criteria, which is 2%, the analysis system for the determination of the assay was verified. The low values of standard deviation denote very good repeatability of the measurement. Thus it was shown that the equipment used for the study were correct and the developed HPLC method was highly repetitive.

The LOD and LOQ tests for the procedure were performed on samples containing very low concentrations of analyses. LOD is

defined as the lowest amount of analyte in a sample that can be detected above baseline noise, but not necessarily quantitated as an exact value. LOQ is defined as the lowest amount of analyte in a sample that can be reproducibly quantitated above the baseline noise, which gives the signal-to-noise ratio (S/N).[26]

Samples should be tested over at least a 48-hour period and quantitation of components should be determined through comparison with freshly-prepared standards. The stability of VRZ in 10 µg/mL standard solutions was determined by storing the solutions at ambient temperature (25±1°C).

The mucoadhesive NP was prepared by adapting the spontaneous emulsification technique previously described by Rençber et al.,[18] which was the avoidance of using toxic organic solvents. This system prolongs the residence time of the dose form at the site of application and facilitates an intimate contact of the dose form with the underlying mucosal surface, and thus contributes to improved and/or better therapeutic performance of the VRZ. The prepared mucoadhesive NP was characterized in terms of PS, PI, ZP, and DL capacity. The mucoadhesive property with two methods of NP was determined. *In vitro* drug release of CSH-coated NP was performed.

The PS of the NP affects its important properties. The most important properties influenced in pharmaceutical technology are increased saturation solubility and adhesiveness to surfaces/membranes. Pharmaceutically positive effects justify the definition of NPs with a size below 1000 nm, and this size limit should be considered when defining a classification system.[27,28] Also, PS has a direct relevance to the stability of the formulation. Larger particles tend to aggregate to a greater extent compared with smaller particles, thereby resulting in sedimentation.[29] The mean size of the prepared mucoadhesive NPs was found as 217.1±4.2 nm. Previously, Mazzarino et al.[30] produced CSH-coated NPs for buccal drug delivery and the PS was found around 200 nm. The PI of the formulation was low (PI <0.5), showing that this method of preparation resulted in highly uniform NP. ZP is an important parameter that is key for the potential stability of colloidal systems. Prepared VRZ loaded mucoadhesive formulation was positively charged, with ZP values with 26.82±0.4 mV. Positive ZP may give rise to a strong electrostatic interaction with a negatively charged mucosal surface.[31] Therefore, we used positively charged NPs for mucoadhesive drug delivery systems as its positively charged surface can be in favour of adhesion to the cell mucosa, which are normally negatively charged.

Two *in vitro* methods were used to demonstrate the interaction between mucoadhesive NP and mucin. Electrostatic interaction is the most expectable mucoadhesive mechanism. After 6 hours of incubation with mucin, there was a decrease in ZP for mucoadhesive NP, which can probably be attributed to interactions between negatively charged sialic groups in the mucin layer and positively charged surface layer of mucoadhesive NP.[27,30] Therefore, it can be concluded that the mucoadhesive NP was able to interact with mucin due to ionic interaction. The turbidity of mucoadhesive NP–mucin aqueous dispersion was examined to obtain information about mucoadhesiveness. The absorbance of the mucin-free dispersion of the NPs did not significantly deviate from 0.4. Changes in the turbidity of mucoadhesive NP with VRZ:mucin dispersion should be considered as an indication for an eventual interaction between NP and mucin, and not due to the motion of particles. The turbidity of mucoadhesive NP–mucin dispersion was higher than that of mucin dispersion itself (Figure 3). This phenomenon could be explained due to the greater thickness of the CSH layer around these particles, as discussed earlier. Yoncheva et al.[32] reported that CSH-coated poly(lactide-co-glycolide) NP–mucin dispersion demonstrated greater turbidity than mucin itself.

Mucoadhesive NPs with VRZ had high DL capacity (~99%). *In vitro* drug release of mucoadhesive NPs in PBS was studied. The drug release from NPs and subsequent biodegradation are important for developing successful formulations. The release rate of NPs depends upon the desorption of the surface-bound/adsorbed drug, diffusion through the NP matrix, diffusion through the polymer wall, NP matrix erosion, and a combined erosion/diffusion process. Thus, diffusion and biodegradation govern the process of drug release.[33] Figure 4 shows that prepared mucoadhesive NP formulation significantly extended the release. No burst effect was observed, indicating that VRZ was homogeneously dispersed in the NP dispersion and that no significant amount of drug was adsorbed onto the NP surface.

The theory of the determination of drug release mechanism from NPs is based on an empirical equation (Ritger & Peppas, 1987).[22] The exponent n has been proposed as indicative of the release mechanism. In this study, the value of n=0.7477 indicates anomalous (non-Fickian) diffusion. Non-Fickian behavior requires two parameters to describe the coupling of diffusion and relaxation phenomena.

CONCLUSION

This study has described the mucoadhesive NP formulation of VRZ using a coating polymer of CSH. The method of prepared NPs was consistent and reproducible, and able to obtain colloidal solutions with adequate PS, PI, ZP, acceptable VRZ loading capacity, and appropriate *in vitro* drug release. The developed HPLC method for VRZ is very simple and specific because all peaks and results confirmed suitable accuracy, specificity, and precision. Therefore, the method could be useful for both routine analytical and quality control assays of VRZ in pharmaceutical formulations. This study has shown that the described mucoadhesive NP formulation containing VRZ is a promising candidate for the local treatment of mucosal diseases. The developed formulation containing VRZ has been found worthy of *in vivo* studies.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Research Foundation of Ege University (12/ECZ/034) for their financial support.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):149-155
DOI: 10.4274/tjps.72692

ORIGINAL ARTICLE

Quantification of Tamsulosin Hydrochloride and Solifenacin Succinate by Discriminative Derivative Synchronous Emission Spectroscopy

Tamsulosin Hidroklorür ve Solifenasin Süksinatın Senkronize Türev Emisyon Spektrometrisi Yöntemiyle Eşzamanlı Miktar Tayinleri

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ABSTRACT

Objectives: The present study was undertaken with the objective to develop and validate a simple spectrofluorimetric method for the simultaneous quantification of tamsulosin hydrochloride and solifenacin succinate.

Materials and Methods: First-derivative synchronous spectrofluorimetry was attempted for the simultaneous quantification of the analytes. Tamsulosin hydrochloride was quantified at a wavelength of 322 nm (zero-crossing wavelength point of solifenacin succinate) and solifenacin succinate was measured at 570 nm (zero-crossing wavelength point of tamsulosin hydrochloride).

Results: Calibration plots were constructed over the concentration range of 2-10 µg/mL for tamsulosin hydrochloride and 30-150 µg/mL for solifenacin succinate. The method gave satisfactory results when it is validated for linearity, specificity, accuracy, precision, LOD and LOQ as per the ICH guidelines. The assay values in the commercial formulation were found to be in the percentage range of 95.0 for tamsulosin hydrochloride and 103.5 for solifenacin succinate by the proposed method. These results were well in agreement with their label claim.

Conclusion: The proposed synchronous analytical method can be employed for routine quality control analysis of tamsulosin hydrochloride/ solifenacin succinate in tablet dose forms.

Key words: Tamsulosin hydrochloride, solifenacin succinate, synchronous spectrofluorimetry, method validation

ÖZ

Amaç: Bu çalışmanın amacı tamsulosin hidroklorür ve solifenasin süksinatın aynı anda miktar tayininin yapılabilmesi için basit bir senkronize spektroflorimetri yöntemi geliştirmek ve valide etmektir.

Gereç ve Yöntemler: Bahsedilen analitlerin aynı anda miktar tayinlerinin yapılabilmesi için birinci türev senkronize spektroflorimetri yöntemi kullanılmıştır. Tamsulosin hidroklorür 322 nm de ölçüm yapılarak (bu dalga boyu solifenasin süksinatın sıfır kesim noktasıdır) solifenasin süksinat ise 570 nm de ölçüm yapılarak (bu dalga boyu tamsulosin hidroklorürün sıfır kesim noktasıdır) miktar tayinleri gerçekleştirilmiştir.

Bulgular: Kalibrasyon eğrileri tamsulosin hidroklorür için 2-10 µg/mL, solifenasin süksinat için ise 30-150 µg/mL konsantrasyon aralığında hazırlanmıştır. Geliştirilen yöntemle; ICH kılavuzlarına göre hesaplanan doğrusallık, seçicilik, doğruluk, kesinlik ve LOD ve LOQ değerleri kullanılarak başarılı sonuçlar elde edilmiştir. Önerilen yöntemle yapılan analiz sonuçları ticari formülasyon içerisinde tamsulosin hidroklorürün %95.0 solifenasin süksinatın ise %103.5 oranında bulunduğunu göstermiştir. Bu sonuçlar preparatın üzerinde belirtilen değerler ile iyi bir uygunluk göstermektedir.

Sonuç: Önerilen analitik yöntemin tablet dozaj formlarında tamsulosin hidroklorür ve solifenasin süksinatın rutin kontrol analizlerinde kullanılabileceği anlaşılmıştır.

Anahtar kelimeler: Tamsulosin hidroklorür, solifenasin süksinat, senkronize spektroflorimetri, yöntem validasyonu

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Received: 13.04.2017, **Accepted:** 13.07.2017

INTRODUCTION

Tamsulosin hydrochloride (TMH) is chemically 5-[(2R)-2-{[2-(2-ethoxyphenoxy)ethyl] amino}propyl]-2-methoxybenzene-1-sulfonamide and used as selective antagonist of α-1A-adrenergic receptors.[1] Solifenacin succinate (SFS) is chemically butanedioic acid (3R)-1-azabicyclo[2.2.2]octan-3-yl (1S)-1-phenyl-1,2,3,4-tetrahydroisoquinoline-2-carboxylate and used as competitive muscarinic acetylcholine receptor (M3) antagonist.[2] Vesomni, a marketed combined tablet dose form of these drugs was used in the treatment of lower urinary tract symptoms associated with benign prostatic hyperplasia.[3] The structures of both TMH and SFS are depicted in Figure 1.

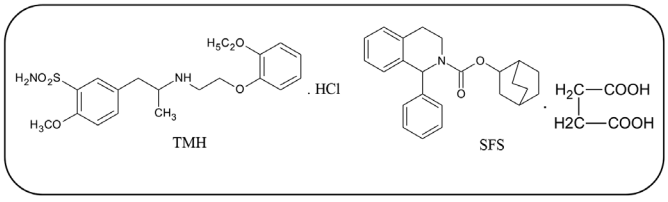

Figure 1. Structures of TMH and SFS

TMH: Tamsulosin hydrochloride, SFS: Solifenacin succinate

A survey of the literature on TMH revealed several methods, such as visible spectrophotometric methods using Folin reagent, sodium nitroprusside-acetaldehyde[4] and bromophenol blue[5], ultraviolet (UV) spectrophotometry[6], fluorimetry using a sodium dodecyl sulphate micellar system[7] and methanol[8]. The methods reported for SFS were mainly based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) and UV spectrophotometry.[9,10] Some spectrophotometric methods using alkaline potassium permanganate, formation of ternary complex with copper (II)/eosin, ammonium molybdate in ammonium thiocyanate and ion-pair complex formation with Bromocresol green[11] have also been described for the analysis of SFS. Spectrophotometric methods are considered to be inappropriate for simultaneous analysis of drugs in multi-component dose forms due to lack of specificity.

To the best of our knowledge, no method has been reported yet for simultaneous quantification of TMH and SFS, except reverse-phase high-performance LC (RP-HPLC).[12] However, chromatographic methods are complex with costly instrumentation, require skilled operators, and expensive solvents limit the application of simultaneous quantification of above the drugs. Spectrofluorimetry has assumed a special status in drug analysis due to its greater sensitivity and specificity than spectrophotometry using two wave-lengths, excitation, and emission. In accustomed fluorescence, an emission spectrum is attained by scanning the monochromator of emission at various wavelengths (λ_{em}), at an appropriate excitation wavelength (λ_{ex}), but in synchronous fluorescence scans both monochromators vary simultaneously. When accustomed spectra are over-lapped, the synchronous technique is used to reduce the extent of overlapping. Derivative spectrofluorimetry is a powerful approach for the resolution of analytes when an analytical peak is overlapped by a large peak of another analyte, particularly in multi-component analysis.[13-21]

The development of a suitable method for simultaneous analysis of TMH and SFS is a challenge because the drugs are present in the ratio of 1:15 in tablet form. If SFS is diluted, the quantification of second drug (TMH) analysis may become difficult. Therefore, simultaneous quantification of TMH and SFS was attempted. A first-derivative synchronous spectrofluorimetric method has been developed based on their native fluorescence and validated as per current International Conference on Harmonization (ICH) guidelines.[22] The emission spectra of TMH and SFS were overlapped hence it was difficult to analyze and quantify their contents by conventional fluorimetry. This overlap needed to be endeavored by copacetic modification, so a first-derivative synchronous spectrofluorimetric method was contemplated.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in the present investigation were of analytical grade. TMH and SFS gift samples were provided by Orchid Pharma Ltd, Chennai, India and the marketed tablet dosage form, Vesomni was procured from local pharmacy.

Instrumentation

The fluorescence spectra and measurements were recorded using a Shimadzu (Japan) RF-5301 PC spectrofluorimeter equipped with 150-Watt Xenon arc lamp, quartz cell (1 cm) and connected to RFPC software. The instrument was operated both at low and high sensitivity with excitation and emission slit width set at 5 nm.

Preparation of standard stock solutions

A stock solution containing 1000 µg/mL of drug was prepared by dissolving 10 mg of TMH/ SFS in 10 mL of distilled water separately. A 1-mL aliquot of stock solution was diluted up to 10 mL with distilled water to attain an end concentration 100 µg/mL of each drug.

Analytical method development

Scanning of drugs by conventional-spectrofluorimetry

TMH or SFS (100 µg/mL) was diluted to 10 mL with distilled water to attain an end concentration of 10 µg/mL. Spectrofluorimetry mode was used for the scanning of the sample against distilled water to obtain the excitation and emission wavelengths, the anon excitation wavelength was fixed, and solutions were further scanned to obtain the emission spectra. TMH exhibited inveterate fluorescence at emission wavelength 328 nm after excitation at 292 nm, similarly SFS exhibited fluorescence at emission wavelength 294 nm after excitation at 256 nm in distilled water. The emission and excitation spectra of TMH and SFS are shown Figure 2 and 3. Though the excitation and emission wavelengths were disparate for both the drugs, and although the fluorescence spectra exhibited protruding intensity, conventional spectrofluorimetry does not permit the simultaneous estimation of both drugs.

Synchronous fluorescence spectra

An attempt was made to attain synchronous spectra of TMH and SFS by maintaining a constant interval of 50 nm between the emission and excitation wavelengths (Figure 4). There was a large overlap of the spectra of TMH and SFS, hence synchronous spectrofluorimetry was found to be inappropriate. This overlap needed to be endeavored by copacetic modification, so first-derivative synchronous spectrofluorimetric method was contemplated.

First derivative synchronous spectrofluorimetry

The synchronous zero-order emission spectra were transformed into consonant first-order spectra in the range of 350-700 nm. Zero crossing criteria can be used for estimations that are proportional to the concentrations of TMH and SFS.

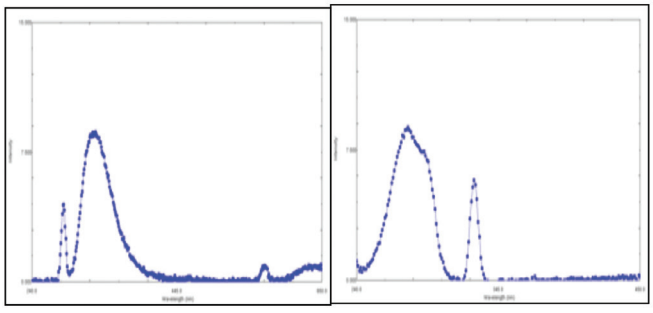

Figure 2. Emission (328 nm) and excitation (292 nm) spectra of TMH (10 µg/mL) in distilled water

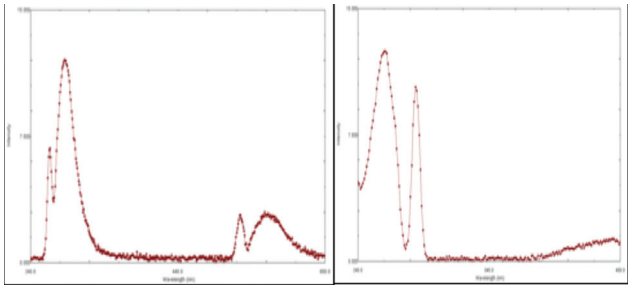

Figure 3. Emission (294 nm) and excitation (256 nm) spectra of SFS (10 µg/mL) in distilled water

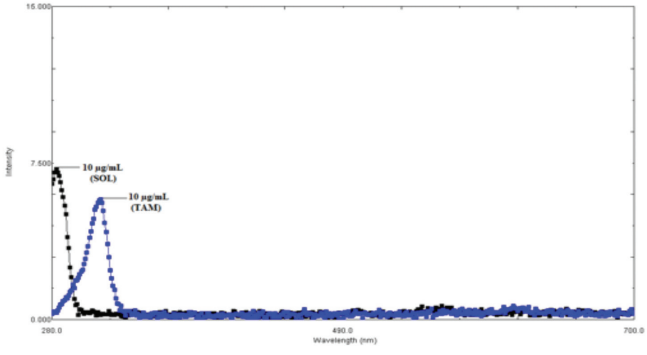

Figure 4. Zero-order synchronous overlaid emission spectra of TMH (10 µg/mL) and SFS (10 µg/mL) in distilled water

TMH: Tamsulosin hydrochloride

Analytical method validation

The method was validated for linearity, specificity, accuracy, precision (both intra and inter-day), limit of detection (LOD), and limit of quantification (LOQ) as per the ICH guidelines to prove that the analytical method could be useful for the quality control of both drugs.

Linearity

The standard concentrations of TMH (2-10 µg/mL) and SFS (30-150 µg/mL) were quantified using first-derivative synchronous spectrofluorimetry, anon fluorescence intensities were recorded and a calibration curve was contrived by plotting the analyte intensities paradoxical to the drug concentrations.

Specificity

The method specificity was assessed by comparing the spectra obtained from the placebo, commercial formulations, and a synthetic mixture of standard solutions. The synthetic mixture was prepared by adding 0.6 mL of TMH standard stock solution (100 µg/mL) and 0.9 mL of SFS standard solution (1000 µg/mL) to a 10 mL volumetric flask, the volume was made up to the mark with distilled water to obtain a final concentration of 6 µg/mL TMH and 90 µg/mL SFS. Then same concentrations of sample solution were prepared using marketed tablets. The method was applied to analyze blank, synthetic mixture, and formulation solutions in order to check if any components of the formulation could generate a response or a read with an emission band similar to the drugs.

Accuracy

Acquisition studies were conducted using a standard addition method where the known amount of TMH/SFS was added to the pre-analyzed sample according to 80, 100 and 120% levels of the label claim, and further subjected to the contemplated analytical method, and anon percentage recovery, and relative standard deviation (RSD%) were calculated for each concentration.

Precision

The intra-day and inter-day precision of the proposed first-derivative spectrofluorimetric simultaneous method was ascertained by estimating the corresponding response three times on the same day (intra-day precision) and on 3 different days over a period of 1 week (inter-day precision) for three different concentrations of TMH (2, 6 and 10 µg/mL) and SFS (30, 90 and 150 µg/mL).The results are reported in terms of RSD%.

LOD and LOQ

The LOD and LOQ for the proposed method were performed on samples containing very low concentrations of analyte (TMH and SFS) as per the ICH guidelines and calculated based on the calibration curve.

Assay of TMH and SFS in their fixed dose formulation

Twenty tablets of marketed formulation (Vesomni) were

accurately weighed and powdered. A quantity of powder equivalent to 0.4 mg of TMH and 6.0 mg of SFS was dissolved in methanol (5 mL) and sonicated for 15 min. The flask was shaken and the volume was made up to 10.0 mL with distilled water. The above solution was filtered through Whatmann filter paper (No.41). From the filtrate, 1.0 mL was transferred into a volumetric flask and the volume was made up to 10.0 mL with distilled water to give a solution containing 4 µg/mL of TMH and 60 µg/mL of SFS. This solution was analyzed using the proposed method for the simultaneous quantification of TMH and SFS. The amount of drugs present in the sample solution were determined by substituting derivative responses into the equation of the linear line representing the calibration curves for TMH and SFS, with correction for dilution.

RESULTS AND DISCUSSION

Analytical method

Synchronous scanning spectrofluorimetry in alliance with derivative techniques is expedient in the locution of sensitivity, spectral discrimination, and decisive identification of chemical species in multi-component analysis. TMH molecules contain two aromatic rings, namely ethoxy phenyl, methoxy benzene-1-sulfonamide and tetrahydroisoquinoline rings, in which greater numbers of π electrons were available to put on view of fluorescence. Different solvent systems were tested in furtherance of the best predicaments, such as the solubility and fluorescence activity of both drugs. TMH and SFS exhibited the indigenous fluorescence at emission wavelength 328 nm subsequent to excitation at 292 nm and emission wavelength 294 nm subsequent to excitation at 256 nm, respectively in distilled water as solvent. The accustomed and synchronous fluorescence spectra of these drugs overlapped substantially, indicating that these methods did not permit the simultaneous determination of both drugs due to this overlap problem, which was overcome by using first-derivative spectrum (Figure 5), revealing that TMH gave zero intensity at 570 nm, whereas SFS gave a significant derivative response, likewise, SFS gave zero intensity at 322 nm, but TMH gave a significant derivative response. Therefore, 322 and 570 nm were elected for the determination of TMH and SFS, respectively, in the synthetic mixture and tablet forms.

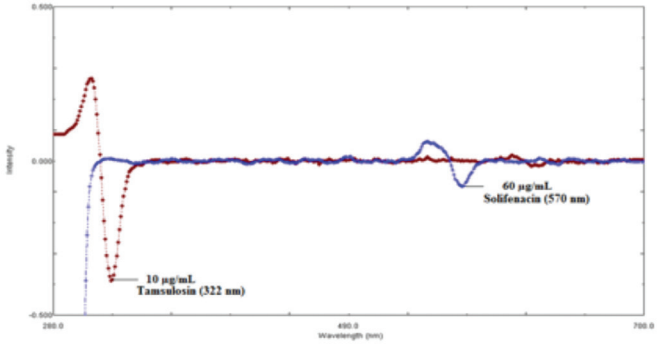

Figure 5. First-order synchronous overlaid emission spectra of TMH (10 µg/mL) and SFS (60 µg/mL) in distilled water

Analytical method validation

Linearity

The calibration curve for TMH and SFS was recorded at 322 and 570 nm, respectively. The linearity was evaluated by the least square regression method. The regression analysis of the calibration curves is shown in Figures 6 and 7. The responses for TMH at 322 nm were found to be linear in the concentration range of 2-10 µg/mL with a correlation co-efficient (r^2) value of 0.9996. Similarly, the responses for SFS at 570 nm were linear in the concentration range of 30-150 µg/mL with a correlation coefficient (r^2) value of 0.9992. From the Figure 8, it can be observed that with the increase in TMH concentration, the derivative response at 322 nm increased. Similarly, the derivative response for SFS at 570 nm was proportional to its concentration.

Specificity

The derivative synchronous spectrum obtained from the commercial formulation solution was compared with the spectrum of synthetic mixture of standard solutions (TMH and SFS) and blank. The spectra of both the commercial formulation and the synthetic mixture were found to be similar. Specificity of the method is shown in Figure 9, which revealed that there was no interference from the excipients in the tablets with derivative response of either drug (TMH and SFS) at their respective analytical wavelengths (322 and 570 nm). Hence, the method was proved to be specific.

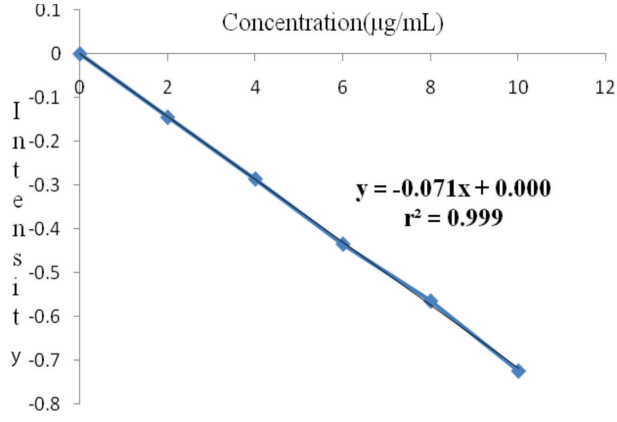

Figure 6. Linearity plot of TMH (2-10 µg/mL) in distilled water at 322 nm

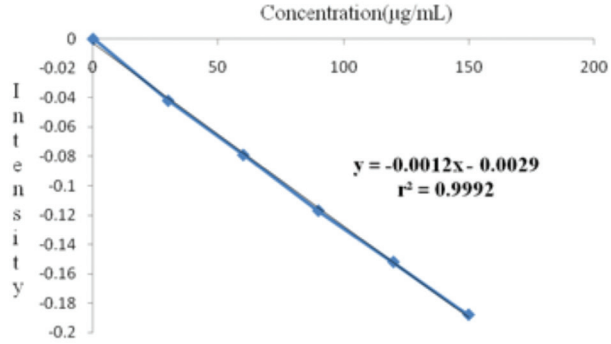

Figure 7. Linearity plot of SFS (30-150 µg/mL) in distilled water at 570 nm

Accuracy

The accuracy of the analytical method was determined using the standard addition method. Three different levels (80, 100 and 120%) of standards were spiked to commercial tablets in triplicate. The mean percentage recoveries and RSD% values were calculated and reported in Table 1. The percentage recoveries of TMH and SFS were found to be in the range 102.27-113.75 and 97.22-101.0, respectively, which were found to be satisfactory.

Precision

The repeatability (intra-day precision) and intermediate precision of the method was determined by three concentrations for both TMH (2, 6 and 10 µg/mL) and SFS (30, 90 and 150 µg/mL). The results are summarized in Table 2. The RSD% of repeatability was less than 2.0 for both drugs, indicating good precision of the developed method.

LOD and LOQ

From the linearity plot the LOD and LOQ of TMH and SFS were calculated. The LOD and LOQ for TMH was 0.210 and 0.639

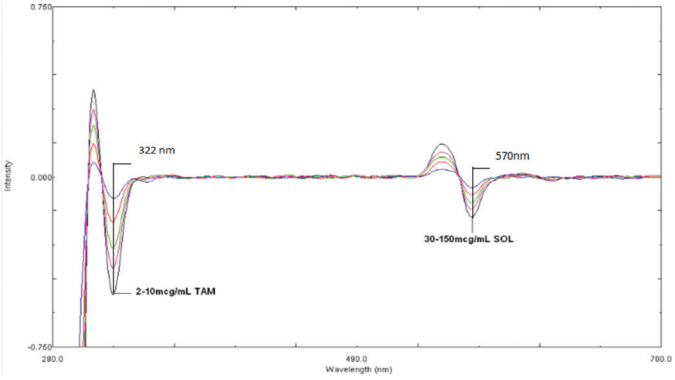

Figure 8. First-derivative linearity spectra of TMH (2-10 µg/mL) and SFS (30-150 µg/mL)

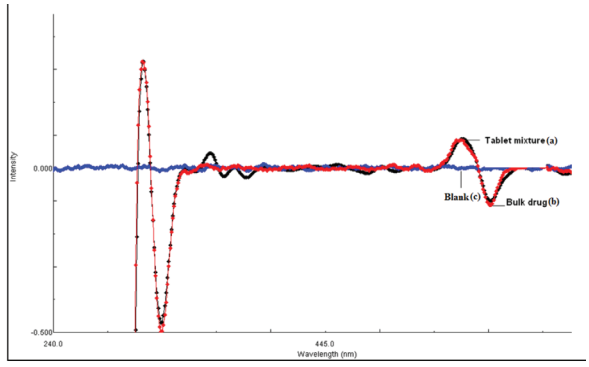

Figure 9. First-order synchronous overlaid spectra of commercial formulation (a), synthetic mixture (b) and blank (c)

Table 1. Accuracy data of the analytical method

Analyte	Recovery level %	Amount of standard (µg/mL)	Conc of sample spiked (µg/mL)	Total amount (µg/mL)	Amount recovery (AM±SD) (µg/mL) (n=3)	% Recovery	RSD%
TMH	80	0.4	0.32	0.72	0.75±0.012	104.16	1.60
	100	0.4	0.4	0.8	0.91±0.007	113.75	0.76
	120	0.4	0.48	0.88	0.90±0.011	102.27	1.20
SFS	80	6.0	4.8	10.8	10.52±0.078	97.22	0.74
	100	6.0	6.0	12.0	12.12±0.026	101.0	0.21
	120	6.0	7.2	13.2	13.00±0.054	98.48	0.41

SFS: Solifenacin succinate, TMH: Tamsulosin hydrochloride, RSD: Relative standard deviation

Table 2. Data for precision of the analytical method

Drug	Concentration (µg/mL)	Intra-day precision		Inter-day precision	
		Concentration estimated (µg/mL) (AM ± SD) (n=3)	RSD%	Concentration estimated (µg/mL) (AM ± SD) (n=3)	RSD%
TMH	2	2.01±0.005	0.24	2.12±0.004	0.18
	6	6.21±0.012	0.19	6.05±0.022	0.36
	10	10.07±0.025	0.24	10.05±0.034	0.33
SFS	30	31.28±0.064	0.20	31.02±0.046	0.40
	90	91.28±0.029	0.03	90.15±0.084	0.09
	150	148.08±0.226	0.15	150.22±0.152	0.10

SFS: Solifenacin succinate, TMH: Tamsulosin hydrochloride, RSD: Relative standard deviation

Table 3. System suitability parameters of TMH and SFS

Parameter	TMH	SFS
Emission wavelength (nm)	322	570
Beer's law limit (μg/mL)	2-10	30-150
Slope (m)	-0.0720	-0.0010
Intercept (c)	0.0002	-0.0030
Correlation coefficient (r^2)	0.9992	0.9996
LOD (μg/mL)	0.210	2.64
LOQ (μg/mL)	0.639	8.0
Regression equation	y=-0.07196X+0.00024	y=-0.00125x-0.00290

SFS: Solifenacin succinate, TMH: Tamsulosin hydrochloride, LOD: Limit of detection, LOQ: Limit of quantification

Table 4. Assay data of TMH and SFS in marketed formulation

Formulation	Drug	Label claim (mg)	Amount found (mg) (AM ± SD) (n=3)	% Assay	RSD%
Vesomni	TMH	0.4	0.38±0.006	95.0	1.57
	SFS	6	6.21±0.024	103.5	0.38

RSD: Relative standard deviation, SFS: Solifenacin succinate, TMH: Tamsulosin hydrochloride

μg/mL and SFS was found to be 2.640 and 8.0 μg/mL. The summary of the system suitability parameters is represented in Table 3. The results obtained from validation evidenced that the proposed method was scientifically sound.

Assay of drugs from commercial tablets

The accuracy of the proposed method was evaluated through the assay of commercially available tablets (VESOMNI) containing 0.4 mg of TMH and 6 mg of SFS. The results obtained were compared with the corresponding labeled amounts and reported in Table 4. The amount of TMH and SFS in formulation was found to be 0.38±0.006 and 6.21±0.024 mg, respectively. The percentage assay in the commercial formulations was found to be 95.0 for TMH and 103.5 for SFS by the proposed method. The RSD% for formulation was less than 2, which indicates the accuracy of the proposed method.

CONCLUSION

A simple and rapid first derivative synchronous spectrofluorimetric method for the simultaneous quantification of TMH and SFS was developed and validated as per the ICH guidelines. The results of validation studies denoted the immense scope of sensitivity, accuracy, precision, and system suitability of the analytical method. The proposed method was successfully adopted for the assay of TMH and SFS and the results were found to be in good agreement with their respective label claims, which suggested that there was no interference of formulation excipients in the estimation. The contemplated spectrofluorimetric method was found to be superior because of its high specificity, spectral discrimination, economic, eco-friendly, and readily available solvent and lack of extraction procedure. These advantages endorse that the developed method can be habitually employed in quality control for simultaneous analysis of TMH and SFS in tablet dose forms.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. C.V.S. Subrahmanyam, Principal, Gokaraju Rangaraju College of Pharmacy and the Gokaraju Rangaraju Educational Society (GRES) for providing necessary laboratory facilities.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):156-165
DOI: 10.4274/tjps.54376

ORIGINAL ARTICLE

Phytochemical Screening for Various Secondary Metabolites, Antioxidant, and Anthelmintic Activity of *Coscinium fenestratum* Fruit Pulp: A New Biosource for Novel Drug Discovery

Coscinium fenestratum Meyvelerinin Farklı Sekonder Metabolitleri Üzerinde Fitokimyasal Analiz Çalışmaları, Antioksidan ve Antihelmintik Aktiviteleri: Yeni İlaçların Keşfinde Yeni Bir Doğal Kaynak

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ABSTRACT

Objectives: *Coscinium fenestratum* (Gaertn.) Colebr. (CF, Family: Menispermaceae) is an important endangered woody climber in India. CF contains various major secondary metabolites for the treatment of various disease conditions. The present study aimed to establish the antioxidant and anthelmintic activity of *Coscinium fenestratum* fruit pulp.

Materials and Methods: The dried fruit pulp was subjected to aqueous, methanol, and mixed aqueous and methanol (1:1) solvent extraction followed by phytochemical investigations, estimations of alkaloids, phenolics, flavonoids, antioxidant potentiality (DPPH and hydrogen peroxide scavenging methods), and anthelmintic activity tests were carried out.

Results: Preliminary phytochemical screening of CF fruit extracts revealed the presence of alkaloids phenols, flavonoids, tannins, steroids, and resins, which are responsible for biologic properties. The combined aqueous and methanol extract resulted in significant anthelmintic and antioxidant properties in a dose-dependent manner. The DPPH free radical scavenging assay and H_2O_2 assay exhibited IC_{50} values of 42.38±0.012 µg/mL and 46.80±0.011 µg/mL, respectively. Thereafter, the anthelmintic activity test was carried out against *Pheretima posthuma* and *Taenia solium* with the extract at varying concentrations of 25, 50, 100 and 150 mg/mL and compared with standard albendazole (25 and 50 mg/mL) and saline (0.9%) as a control. All the extracts exhibited concentration-dependent paralytic effect, followed by death on the test organism, but significant activity was observed with the combined methanol and aqueous extract.

Conclusion: The study was conducted in order to find possible isolated compounds as a biosources for future novel antioxidants in food and pharmaceutical formulations. Our findings indicate for the first time that the CF fruit pulp has therapeutic values with prominent antioxidant and anthelmintic properties.

Key words: Antioxidant study, anthelmintic activity, *Coscinium fenestratum*, extracts, phytochemical study

ÖZ

Amaç: *Coscinium fenestratum* (Gaertn.) Colebr. (CF, Familya: Menispermaceae) Hindistan'da önemli bir odunsu bitkidir. Bitki çeşitli hastalıkların tedavisinde etkili olan çeşitli majör sekonder metabolitler içermektedir. Bu çalışmada, *Coscinium fenestratum* meyvelerinin antioksidan ve antihelmintik aktiviteleri değerlendirilmiştir.

Gereç ve Yöntemler: Bu çalışmada, kurutulmuş meyvelerin sulu, metanollü ve sulu metanollü (1:1) ekstreleri hazırlanmış ve ekstrelerin alkaloit, fenolik, flavonoid içerikleri ile antioksidan (DPPH ve hidrojen peroksit süpürücü etki tayin yöntemi) ve antihelmintik aktiviteleri değerlendirilmiştir.

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Received: 12.05.2017, Accepted: 03.08.2017

Bulgular: Ön fitokimyasal tarama çalışmaları, CF meyve ekstrelerinin biyolojik etkiden sorumlu alkaloitler, fenoller, flavonoidler, tanenler, steroidler ve reçine içerdiğini ortaya koymuştur. Sulu metanollü ekstrenin doz bağımlı olarak anlamlı derecede antihelmintik ve antioksidan etkiye sahip olduğu belirlenmiştir. DPPH serbest radikal süpürücü aktivite tayini ve H_2O_2 deneyinde IC_{50} değerleri sırasıyla 42.38±0.012 µg/mL ve 46.80±0.011 µg/mL olarak belirlenmiştir. Antihelmintik aktivite, *Pheretima posthuma* ve *Taenia solium*'a karşı ekstrenin 25, 50, 100 ve 150 mg/mL gibi değişen konsantrasyonlarında, standart albendazol (25 and 50 mg/mL) ve kontrol olarak kullanılan tuzlu suya (%0.9) kıyasla denenmiştir. Tüm ekstreler doz bağımlı paralitik etki göstermiş, organizmalar üzerinde anlamlı derecede öldürücü etki, sulu metanollü ekstre için tespit edilmiştir.

Sonuç: Bu çalışmanın bulguları, CF meyve ekstresinin antioksidan ve antihelmintik etkileri ile tedavi edici etkiye sahip olduğunu ilk defa göstermiş, izole edilen bileşiklerin gıda ve farmasötik formülasyonlarda yeni antioksidan doğal kaynaklar olarak kullanılabileceğini ortaya koymuştur.

Anahtar kelimeler: Antioksidan çalışma, antihelmintik etki, *Coscinium fenestratum*, ekstreler, fitokimyasal çalışma

INTRODUCTION

Secondary metabolites are important plant constituents for effective therapeutic activities. It was reported that the presence of this specific group of compounds showed specific medicinal actions and sometimes traditionally reported, but there is little by way of scientific validation. Among these activities, antioxidant and anthelmintic activities are very important. Rapid production of free radicals leads to oxidative damage to biomolecules and results in serious disorders viz. degenerative disorders, cancer, diabetes, neural disorders, and ageing, and hence antioxidant plays a vital role to block free radical production.[1,2] Moreover, infections with parasitic worms are serious problems for humans, producing various diseases worldwide, and helminthes is one of them. There are various types of worms viz. round worms or nematodes including intestinal worms, filarial worms that cause lymphatic filariasis, chocerciasis, platyhelminths or flatworms including flukes, and tapeworms.[3] These worms cause lymphatic filariasis, onchocerciasis, cysticercosis, malnutrition, anemia, eosinophilia, and pneumonia, which are life-threatening. As per the report of the World Health Organization, more than two billion people have parasitic worm infections globally.[4] Treatment with synthetic drugs causes many adverse effects and helminthes become resistant. Hence, there are demands for natural plant secondary metabolites in the treatment and prevention of this chronic problem.

Coscinium fenestratum (Gaertn.) Colebr. (CF) is a woody climber that belongs to the family Menispermaceae. The plant is commonly known as tree turmeric or false Calumbadue due to its yellow stem. CF is found in Asian countries such as India, Malaysia, Vietnam, Myanmar, Singapore, Thailand, and Sri Lanka.[5,6] In India, the plant is endangered and located in the Western Ghats areas, especially in high rainfall evergreen forests of Karnataka, Kerala, and Tamil Nadu at altitudes of 500-750 m.[7,8] The tree requires long seed germination times and takes 14-15 years to mature and flower. Hence, the fruits and seeds are very rare and this leads to endangered red- labeled species due to over exploitation from natural habitats, zero cultivation planning, and trees being uprooted before their reproduction stage for their medicinal importance.[4] The leaves and roots are traditionally used for the treatment of ulcers, skin diseases, eye disorders, inflammation, hypertension, jaundice, diabetes, and snake bites.[9-11] Multiple beneficial pharmacologic-related properties with various solvent extracts of the leaves and roots of CF have been reported viz. hepatoprotective, immune protective, hypoglycemic, anti-tumor activities, dressing wounds, ulcer treatment, and for cutaneous leishmaniasis, and it is non-toxic to mammals.[12,13] Stem and root extracts have also shown antioxidant and antimicrobial potential.[14] Traditionally CF is used as one of the ingredients in several ayurvedic preparations such as soap, bath gels, face wash and bath oil, and in the cosmetic industry as facial masks, fairness creams, and body lotions.[4] Furthermore, stem extract of CF was reported to have a significant effect on stimulating insulin secretion.[15] These activities are due to the presence of the important alkaloid-containing phytoconstituents such as berlambine, dihydroberlambine, noroxyhydrastine, berberine(an isoquinoline alkaloid) and other constituents such asceryl-alcohol, saponin, hentriacontane, sitosterol glucoside, palmitic acid, and oleic acid, which are isolated from the stem and roots of the plants.[16] Recently ecdysterone was identified and isolated from the stem and leaves of CF and evaluated using high-performance liquid chromatography and liquid chromatography-mass spectrometry.[17] The availability of various phytoconstituents in fruits is unknown and hence it is required to select the various solvent extractions for the fruits for further processing. It was reported that the constituents varied with solvent extraction and the zone of collection of the raw materials.[18-20] Till now, no literature has revealed the medicinal importance of the fruit and the pulp constituents, perhaps due to improper collection or the low availability of the fruits and seeds. It is also essential to understand the cause of delayed germination. Based on the availability of secondary metabolites in the plant, the present study was conducted to determine and evaluate the phytoconstituents present and novel antioxidant and anthelmintic activities were assessed for the first time from various extracts of the dried fruit pulp.

MATERIALS AND METHODS

Collection and identification of fruits

One hundred CF fruits were collected from Dr. Gokul S, CIMAP Research Centre, Allalasandra, GKVK Post, Bangalore -65 (Latitude: 12° 58' N and Longitude: 77° 38' E), and authenticated by Dr. P.E. Rajasekharan, Principal Scientist, IIHR, Bangalore. The fruits are stored as herbarium in Pharmacognosy Department of Krupanidhi College of Pharmacy, Bangalore (Herbarium No: CF-317/KCP/2016-17).

Morphological study of fruits and seeds

Fifty fruits were randomly selected and measured for diameter using *Vernier calipers* with the measurement readings in centimeters (cm), with precision up to 2 decimal places.

Thereafter, seed diameters were also measured after removal of pulp and resinous matter to understand the probability for late germination. In addition, a complete morphologic study was performed, observing color, odor, size, shape, and extra features.

Preparation of extracts

Fresh fruits of the CF plant were shade-dried for several days and in between observed for fungal infections. The dried pulps were ground to a course powder and 250 g of the same underwent soxhlet extraction with light petroleum ether for 4 hrs and defatting the materials. The pulps were successively extracted with four solvents viz. chloroform, methanol (80%), aqueous, and an equal ratio mixture of aqueous and methanol (80%) (1:1). The reflux method was used for all extracts separately for 7-8 hrs (after drying after each extraction) preparation and finally the yield was calculated after removal of the solvents by rotary evaporation (at 45˚C) and the dried extract was stored in a refrigerator (at 4-5˚C) for further investigations.

Phytochemical screening

The preliminary phytochemical analysis of the plant extracts was performed using the standard protocol as describe by Khandelwal[21], and Kokate[22], to identify the presence of alkaloids, flavonoids, steroids, glycosides, cardiac glycosides, anthraquinones, tannins, and saponins.

Based on the presence of phytoconstituents, the following estimations of secondary metabolites were carried out:

Determination of alkaloid

Onemilligram of each plant extract was dissolved separately in dimethyl sulphoxide and 1 mL of 2 N HCl was added and filtered. The solutions were transferred to a separating funnel with the addition of 5 mL of Bromocresol green solution and 5 mL of phosphate buffer. The mixture was then shaken thoroughly with 1, 2, 3, and 4 mL of chloroform and collected in a 10-mL volumetric flask and diluted to volume with chloroform. A set of reference standard solutions of atropine (10, 20, 30, 40, 50 and 60 µg/mL) were prepared in the same manner. The absorbances for the test and standard solutions were determined using an ultraviolet (UV) spectrophotometer at 470 nm. A blank sample was prepared for error correction. Finally, the total alkaloid content was calculated as mg of atropine equivalent (AE)/g of each extract.[23]

Total phenolic content

The total phenolic compounds in all three fruit pulp extracts of CF were determined using Folin-Ciocalteu's method. A blue color is formed during the reaction, which is measured spectrophotometrically.[24] One milliliter of sample (1 mg/mL) was mixed with 1 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 10 mL of a 7% Na_2CO_3 solution was added to the mixture, then 13 mL of deionized distilled water was added and mixed thoroughly. The mixture was kept in the dark for 90 min at 23˚C for the reaction (blue color formation). Gallic acid was used as a standard and the standard solution was prepared as per the same method followed for the sample (10, 20, 30, 40, 50 and 60 µg/mL). Then, absorbance was taken at 765 nm. The total phenolic content was determined from extrapolation of a calibration curve, which was made by preparing a gallic acid solution, and was expressed as mg of gallic acid equivalent (GAEs) per g of extract (GA mg/g). The estimation of the phenolic compounds was carried out in triplicate. The following formula was used for the calculation:

$T = (C \times V)/M$

Where, T = total content of phenolic compounds, mg/g plant extract, in GAE; C = concentration of gallic acid established from the calibration curve (µg/mL); V = volume of extract (mL); M = weight of water extract of the plant (g).

Total flavonoid content

The total flavonoid content was measured using an aluminum chloride colorimetric assay as described by Park et al. [25] In a 10-mL test tube, 0.3 mL of extracts, 3.4 mL of 30% methanol, 0.15 mL of $NaNO_2$ (0.5 M), and 0.15 mL of $AlCl_3.6H_2O$ (0.3 M) were mixed thoroughly. After a few min, 1 mL of NaOH (1 M) was added. In the same way, the standard solution was also prepared using rutin (Ru) as a standard. The standard curve for total flavonoids was made using a Ru standard solution (10, 20, 30, 40, 50 and 60 µg/mL). The solution was mixed well and the absorbance was measured against the reagent blank at 506 nm. The total flavonoids were expressed as mg of Ru equivalent/g of dried extract.

Antioxidant assays

Each sample was dissolved in 80% methanol to make a concentration of 1 mg/mL and then diluted to prepare the series concentrations for antioxidant assays.

1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay

All the fruit pulp extracts were tested for their free radical scavenging activity against the stable free radical DPPH. The ability to bleach DPPH by the extracts was quantified using a spectrophotometer. The method used was as described by Brand-Williams et al.[26] One milliliter of 0.1 mM DPPH solution in methanol was mixed with 1 mL of plant extract solution of varying concentrations (25, 50, 100, 150, and 200 µg/mL). Corresponding blank samples were prepared using a mixed 1 mL methanol and 1 mL DPPH solution, methanol and L-ascorbic acid was used as reference standard (1-100 µg/mL) and the experiment was performed in triplicate. The decrease in absorbance was measured at 517 nm after 30 min in the dark (for the reaction) using a UV-Vis spectrophotometer. The percentage scavenging was calculated using the following formula:

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

The IC_{50} value of the sample i.e. the concentration of sample required to inhibit 50% of the DPPH free radical, was calculated using the calibration curve by linear regression.

Hydrogen peroxide-scavenging activity:

The hydrogen peroxide assay was as described by Nabavi et al.[27] Hydrogen peroxide solution (2 mmol/L) was prepared in phosphate buffer (pH 7.4). Extract (0.6 mL) at various

concentrations (25, 50, 100, 150 and 200 µg/mL) was added to hydrogen peroxide solution. For each concentration, a separate blank sample was prepared. The absorbance of hydrogen peroxide with a UV visible spectrometer at 230 nm was determined after 10 min, and then readings were calculated for the blank solution containing phosphate buffer without hydrogen peroxide. The percentage inhibition of H_2O_2 scavenging activity was calculated using the formula below:

% Scavenging activity = [1- (Absorbance of test / Absorbance of control)] x 100

Anthelmintic property

Chemicals and drugs

All chemicals and drugs were obtained commercially and were of analytical grade. Albendazole was purchased from the local market of Bangalore. Dimethyl formamide (DMF) were purchased from Merck, Germany.

Selection of experimental organisms

The preliminary assay was performed on adult earthworm, *Pheretima posthuma*, belong to class Oligochaeta. Due to its easy availability and its anatomic and physiologic resemblance with intestinal round worm parasites of humans, they have been widely used for preliminary evaluations of anthelmintic activity. The experiment was conducted after ethics clearance was obtained from the Institutional Animal Ethics Committee of Krupanidhi College, Bangalore (Approval no: KCP/PCOL/5/2017/). Thereafter, tapeworm (*Taenia solium*, Family: Taeniidae) was selected for assurance of anthelmintic activity. Earth worms were collected from moist soil of the medicinal garden of Krupanidhi College of Pharmacy, Bangalore, and tape worms were collected from a local slaughter house (infested intestines of pigs), Yeshwanthpur, Bangalore. Both were separately washed with normal saline to remove all foreign matter from the body and later used for the anthelmintic study. Earthworms of 3-5 cm in length and 0.1-0.2 cm in width, and tapeworms of 6-8 cm in length were used for the entire experimental protocol. Albendazole (25 mg/mL and 50 mg/mL) was used as a standard solution (prepared by dissolved in DMF) and each test solution of the CF fruit extracts (25, 50, 100 and 150 mg/mL) was evaluated for anthelmintic activity.

Methods

For the evaluation of each plant extract, four worms were placed in separate Petri dishes containing 20 mL solution of crude extracts in the said concentrations and then the worms were introduced to the solutions. The same method was used for each case.

Observations

Observations were made for the time taken for paralysis and death of individual worms during the completion of the investigation. When there was no movement of any part of the body, the time was noted for the paralysis condition, followed by the death time, which was noted when no movement of any part of the body even after being shaken vigorously, and also followed by fading of the body colors of the worms. Death was also ascertained when the worms were dipped in warm water at 50°C.[28] The experiment was carried out as per the guidelines of the Institutional Biosafety and Ethics Committee.[29]

Statistical analysis

Data are expressed as mean ± SD from three replications. For antioxidant assays and anthelmintic activity, the one-way ANOVA test followed by Tukey's test ($p<0.05$) was used to analyze the differences among IC_{50} of the various extracts for different antioxidant assays. The IC_{50} values were determined using the Graph Pad Prism 5 software. Correlation coefficient (r) was calculated for the extract and the activities. P values less than 0.05 were considered statistically significant.

RESULT AND DISCUSSION

Morphologic study of the fruits and seeds

Vernier calipers were used to measure the diameter (50 measurements) of CF fruits and seeds separately (Figures 1 and 2) and the diameters were recorded (Table 1 and 2, respectively). The color of the fruits (Figure 3) was greyish ash and there was no odor. The sizes ranged from 10-14 cm in diameter and the shape was globulus, tapering towards the embryonic site. Each fruit contained a single seed. The fruit pulp was made up of resinous matter (Figure 1). In contrast, the color of the seeds was off-white to gray; the seeds also had no odor. The seeds were 4-6 cm in diameter and sub globular-

Figure 1. Measurement of CF fruit

Figure 2. Measurement of CF seed

shaped, divaricate. The seeds were very hard to break (Figure 4). The high content of alkaloids i.e. berberine in the seed and fact that the embryonic opening part was closed by resinous matter were considered to cause the delayed germination. The average diameter of fruit was 12.35 cm; the minimum diameter of fruit was 10.6 cm; and the maximum diameter of fruit was 13.7 cm. The average seed diameter was 5.03 cm; the minimum seed diameter was 4.1 cm; and the maximum seed diameter was 6.3 cm.

Figure 3. Fruits of CF

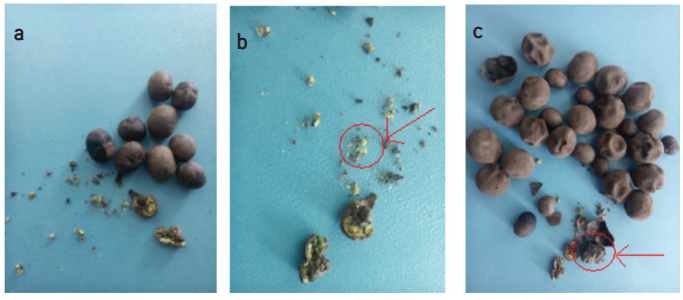

Figure 4. Seeds of CF a) Seeds without resinous mass, b) Broken seed with yellow berberine, c) Seeds surrounded by resinous mass

Extract yield:

The yield and color of the crude extract obtained from the extracted fruit pulp of CF are depicted in Table 3 and Figures 5 and 6. The yield of the extracts was found at a higher percentage (5.76%) in the combined extract of methanol and aqueous solvents, followed by the methanol (5.12%) and aqueous (4.12%) extracts. Earlier literature are also reported that combined extracts increased the yield of the crude extracts more than the individual extract in particular plant species.[19,30]

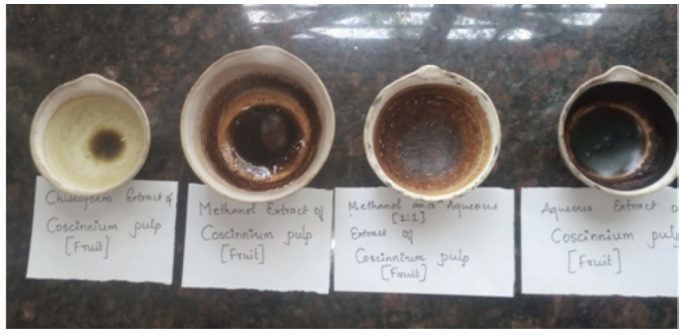

Figure 5. Various CF fruit pulp extracts

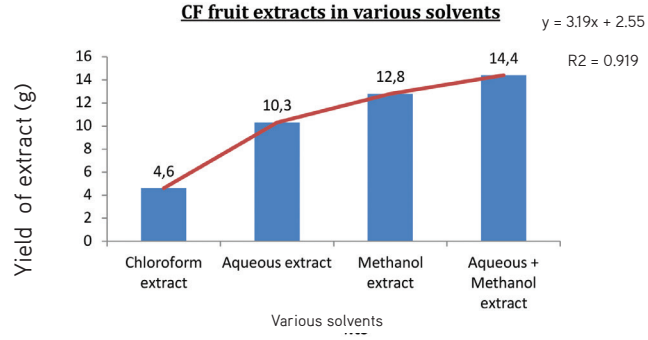

Figure 6. Yield of crude extracts of CF fruits in various solvents

Table 1. CF Fruit diameter (50 measurements) determination

Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)
1	12.5	11	12.4	21	12.4	31	12.6	41	11.7
2	11.7	12	12.7	22	12.6	32	13.2	42	11.4
3	13.2	13	12.3	23	13.7	33	13.2	43	10.9
4	12.7	14	11.5	24	13.2	34	11.5	44	10.9
5	12.6	15	11.3	25	13.5	35	11.5	45	12.4
6	13.1	16	13.2	26	11.6	36	11.4	46	12.5
7	13.4	17	11.8	27	11.5	37	12.4	47	10.6
8	11.6	18	13.6	28	11.8	38	12.5	48	11.8
9	11.4	19	13.2	29	12.1	39	12.5	49	11.6
10	12.1	20	13.6	30	13.6	40	13.4	50	13.7

Each fruit diameter was determined using Vernier calipers

Table 2. CF Seed diameter (50 measurements) determination

Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)	Sl No.	Diameter (cm)
1	4.1	11	5.3	21	5.3	31	4.6	41	4.8
2	4.7	12	5.5	22	5.2	32	4.5	42	6.1
3	5.6	13	4.7	23	5.1	33	4.5	43	6.1
4	4.3	14	4.5	24	4.4	34	4.5	44	5.6
5	4.1	15	4.6	25	4.5	35	6.2	45	5.6
6	5.3	16	4.2	26	4.3	36	5.5	46	5.3
7	5.4	17	5.3	27	4.7	37	5.4	47	4.9
8	5.6	18	6.1	28	4.7	38	5.3	48	4.7
9	4.2	19	6.3	29	4.8	39	5.2	49	4.4
10	5.3	20	5.6	30	5.2	40	5.4	50	4.3

Each seed diameter was determined using Vernier calipers

Table 3. Yield and color of extracts of CF fruit pulps

Different extracts	% Yield	Color of extract
Chloroform	1.84	Pale greenish Ash
Methanol (80%)	5.12	Medium to dark brown
Aqueous	4.12	Dark brownish grey
Aqueous + methanol (80%) (1:1)	5.76	Light to medium dark brown

Table 4. Various chemical tests for CF fruit pulp extracts

Tests	Different extracts			
	Chloroform	Methanol	Aqueous	Aqueous + methanol (1:1)
Protein	+	--	--	--
Carbohydrate	--	--	--	--
Lipid	+	--	--	--
Alkaloids	--	+	+	++
Glycosides	--	+	+	++
Tannins	--	+	+	++
Flavonoids	--	+	--	++
Saponin	--	+	+	+
Resin	+	+	--	+
Steroids	--	--	--	--
Phenols	--	+	+	++

(--) = Negative test; (+) = Positive test

The present investigation also resulted similarly. This indicated that the CF fruit pulp extracts was also dependent on the type of solvent used. A literature survey revealed that the extraction yield increases with the increasing polarity of the solvent used in extraction. Hence, the combined use of water and organic solvent may facilitate the extraction of soluble chemicals in water and/or organic solvent, and accordingly, the yield of secondary metabolites is higher than with individual solvent extraction.[31] The results of this study are in agreement with the extraction yields of some medicinal plants.[32,33]

Table 5. Content of total alkaloids in CF fruit pulp extracts

Extracts	Alkaloid content (mg of AE/g)
Methanol extract	68.20±0.025**
Aqueous extract	41.01±0.015**
Aqueous + methanol extract	77.02±0.020**

Mean ± SD (n=3); One-way ANOVA study followed by Tukey's post test. Significance level, **p<0.05. AE: Atropine equivalent

Phytochemical screening

Various chemical tests were performed to detect the presence of secondary metabolites. The results are tabulated in Table 4. Based on the availability of the secondary metabolites, further estimation of the plant's vital constituents viz. total alkaloids, phenols, and total flavonoid were determined for methanol, aqueous, and combined aqueous and methanol extracts.

Total alkaloids

The alkaloid content was determined in CF fruit pulp extracts and expressed in terms of AE as mg of AE/g of extract (standard curve equation: $y = 0.014x + 0.106$, $R^2 = 0.994$). The highest concentration of alkaloid was measured as 77.02 mg/g in the combined extract. This is because of the high solubility of the alkaloids in the combined extract rather than in individual extracts (Table 5).[31,33]

Total phenolic content

The total phenolic content in the entire extracted CF fruit pulp was determined using Folin-Ciocalteu's reagent and is expressed in terms of GAEs (mg of GA/g of extract, standard curve equation: $y = 0.012x + 0.166$, $R^2 = 0.991$). The highest

concentration of phenols was measured in the combined aqueous and methanol extract, followed by the methanol and aqueous extract. It was reported that high solubility of phenols in polar solvents provides high concentrations in extracts,[34,35] and the same trend followed in the present investigation where combined aqueous and methanol solvents increased the solubility of phenolic compounds more than with individual solvents (Table 6).

Total flavonoid content

The concentration of flavonoids was determined using spectrophotometry for all three extracts and the content of flavonoids is expressed in terms of Ru equivalent (mg of Ru/g, the standard curve equation: y = 0.011x + 0.041, R^2 = 0.993). In this case, the same trend also followed as above. The highest flavonoid concentration (98.03 mg Ru/g) was recorded for the combined extract, followed by the methanol extract. This result was due to the solubility. It was reported that the concentration of flavonoid in plant extracts depends on the polarity of solvents used in the extract preparation.[36] A similar result was obtained in this study (Table 7).

Based on the estimation of total alkaloids, phenols, and flavonoids, a further investigation was carried out to reveal an antioxidant study. Phenolic and flavonoid compounds are known to have a correlation with antioxidant activities.[37,38] The present study revealed the higher content of these compounds, and due to their presence, the CF fruit pulp may have scavenging activity i.e. mainly due to their redox properties, which play an important role in adsorbing and neutralizing free radicals and decomposing peroxides.[39] In view of this, two different methods were used to reveal antioxidant activity.

Antioxidant assays

An antioxidant is defined as any substance that inhibits oxidative damage to a target molecule.[40] Antioxidant compounds such as phenolic acids, polyphenols, and flavonoids reduce free radicals such as peroxide, hydrogen peroxide or lipid peroxyl, and thus inhibit oxidative mechanisms that lead to degenerative diseases.[41] Based on that, the following few methods were studied for the CF fruit pulp extract.

DPPH: The free radical scavenging activity of all crude extracts of the CF fruit pulp was quantitatively determined using a DPPH assay along with IC_{50} values. IC_{50} values represent the particular concentration of a test extract that inhibit activity by 50%. The results are tabulated in Figure 7. The effect of antioxidants on DPPH is thought to be due to their hydrogen-donating ability.[42] DPPH is a purple-colored dye with absorption maxima of 517 nm, and upon reaction with a hydrogen donor, the purple color fades or disappears due to its conversion to 2, 2-diphenyl-1-picryl hydrazine, and hence absorbance is decreased. The combined aqueous and methanol extracts showed the maximum percentage inhibition (68.43%), followed by the methanol extract (58.24%) at 200 µg/mL concentration, whereas L-ascorbic acid showed 92.17% inhibition. The scavenging activity potency for all the extracts was determined using IC_{50} values. The combined extract (aqueous + methanol) showed a lower IC_{50} value of 42.38 µg/mL, followed by the methanol extract (52.43 µg/mL, Table 8) when compared with standard L-ascorbic acid (4.87 µg/mL).

Hydrogen peroxide radical scavenging assay: Hydrogen peroxide is a weak oxidizing agent, and through oxidation of essential thiol (-SH) groups, it can inactivate a few enzymes. The generation of H_2O_2 in minimum quantities in biologic systems is significant to determine. Naturally-occurring iron complexes inside the cell react with H_2O_2 *in vivo* and highly reactive hydroxyl radicals are generated, which cause the origins of toxic effects.[43]

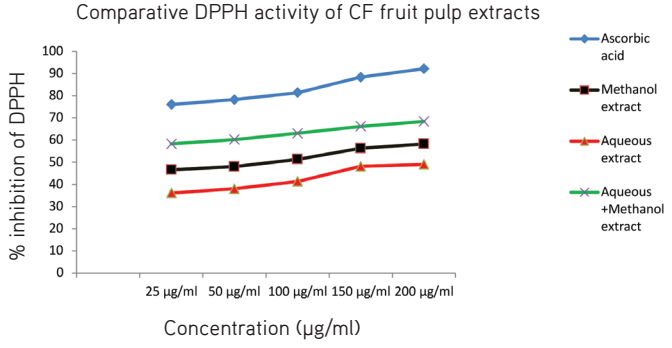

Figure 7. DPPH activity of L-ascorbic acid and various CF fruit extracts
Results were triplicate, each values represent mean ± SD (n=3)

Table 6. Content of total Phenolics in CF fruit pulp extracts

Extracts	Phenolic content (mg of GA/g)
Methanol extract	56.12±0.015**
Aqueous extract	43.22±0.020**
Aqueous + Methanol extract	69.13±0.188**

Mean ± SD (n=3); One-way ANOVA study followed by Tukey's post test. Significance level, **p<0.05. GA: Gallic acid

Table 7. Content of total Flavonoids in CF fruit pulp extracts

Extracts	Flavonoids content (mg of RuE/g)
Methanol extract	36.02±0.021**
Aqueous extract	24.12±0.020**
Aqueous + methanol extract	48.22±0.015**

Mean ± SD (n=3); One-way ANOVA study followed by Tukey's post test
Significance level, **p<0.05. RuE: Rutin equivalent

Table 8. IC_{50} values of different extracts of CF fruits in DPPH and H_2O_2 scavenging assay

Extracts	IC_{50} µg/mL	
	DPPH scavenging assay	Hydrogen peroxide-scavenging activity
Methanol	52.43±0.024	54.22±0.014
Aqueous	66.21±0.021	68.28±0.031
Aqueous + methanol	42.38±0.012	46.80±0.011
Ascorbic acid (standard)	4.87±0.030	9.18±0.020

Results of triplicate tests, Each value represents mean ± SD (n=3)

The scavenging activity of CF fruit extracts was evaluated and compared with ascorbic acid and the results are tabulated in Figure 8. It was reported that H_2O_2 scavenging activity of extracts depends on the phenolic content, which can donate electrons to H_2O_2;[44] the present study revealed the content of phenolics showing scavenging activity of H_2O_2. Among the three extracts, the combined extract (aqueous + methanol) followed by the methanol extract showed good activity in depleting H_2O_2 with IC_{50} values of 46.80 and 54.22 µg/mL, respectively (Table 8). The percentage of H_2O_2 scavenging activity of the combined extract was found as 61.07%, followed by the methanol extract (59.23%) at 200 µg/mL concentration as compared with standard L-ascorbic acid (90.13%).

Correlation matrix: There is a direct correlation between the percentage yield and the content of secondary metabolites; the antioxidant study was observed in the present investigation.

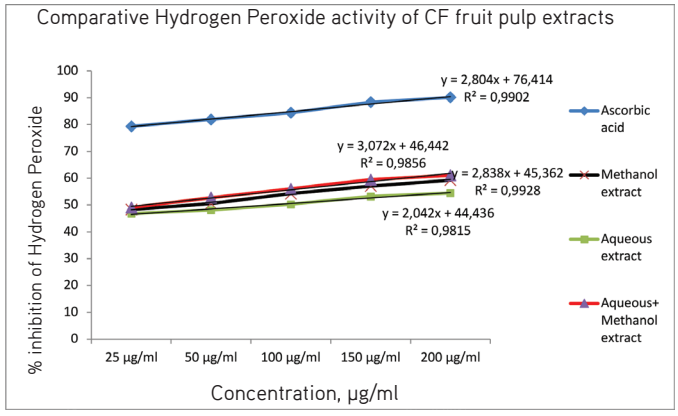

Figure 8. Hydrogen peroxide scavenging activity of L-ascorbic acid and various CF fruit extracts

Table 9. Correlation matrix among the percentage yield, content of secondary metabolites, and antioxidant parameters

Parameters	% Yield	Total phenolic content	Total alkaloid content	Total flavonoid content	% inhibition of DPPH	% inhibition of H_2O_2
% Yield	1.00					
Total phenolic content	0.992**	1.00				
Total alkaloid content	0.987*	0.957	1.00			
Total flavonoid content	0.991	0.999**	0.957	1.00		
% inhibition of DPPH	0.988*	0.999**	0.951	0.999**	1.00	
% inhibition of H_2O_2	0.992*	0.968	0.999**	0.967	0.961	1.00

**Significant at 1%; *Significant at 5%

Table 10. Anthelmintic activity of various extracts of CF fruit pulp

Extracts	Concentration (mg/mL)	Earth worms		Tape worms	
		Time taken for paralysis (min)	Time taken for death (min)	Time taken for paralysis (min)	Time taken for death (min)
Control (0.9% normal saline)	---	---	---	----	----
Methanol extract	25	34.03±0.04*	41.01±0.21*	36.22±0.11*	42.23±0.01*
	50	30.11±0.04*	38.19±0.14*	33.23±0.12*	40.02±0.34*
	100	26.06±0.04*	36.03±0.11*	28.13±0.11*	38.01±0.01*
	150	22.07±0.04*	28.04±0.02*	24.15±0.12*	29.02±0.02*
Aqueous extract	25	39.21±0.01*	48.13±0.11*	40.31±0.20*	45.02±0.21*
	50	35.31±0.05*	43.06±0.04*	37.42±0.01*	42.10±0.24*
	100	29.01±0.05*	37.07±0.11*	32.33±0.12*	39.11±0.21*
	150	26.20±0.11*	30.02±0.05*	28.31±0.11*	35.22±0.15*
Aqueous + methanol extract (1:1)	25	33.10±0.07*	42.20±0.11*	34.20±0.03*	45.10±0.21*
	50	29.11±0.04*	38.21±0.01*	30.13±0.01*	41.16±0.01*
	100	24.20±0.12*	32.12±0.04*	26.21±0.10*	33.11±0.10*
	150	19.12±0.24*	26.01±0.01*	21.14±0.22*	27.32±0.01*
Albendazole (standard)	25	23.13±0.10	29.03±0.02	22.01±0.31	26.01±0.21
	50	18.30±0.11	25.20±0.20	19.20±0.10	28.11±0.01

Values are expressed as mean ± SD. Values were find out by using one-way ANOVA followed by Dunnett's t-test. *Values are significantly different from control at ($p<0.05$)

The results are depicted in Table 9.

The results from Table 9 indicate that the percentage yield of extract in particular solvents (CF fruit pulp) has a direct correlation with the content of secondary metabolites and even antioxidant activities. Furthermore, Table 8 indicates that antioxidant activity is dependent on IC_{50} values, which are inversely correlated; this result is in agreement with earlier research findings.[45] Based on the presence of various phytochemicals viz. alkaloids, phenols, tannins, flavonoids and due to the strong antioxidant activity of CF fruit extracts, further anthelmintic activity was tested for the first time. Many scientific studies have already revealed that the presence of phenols, tannins, and flavonoids leads to anthelmintic activity.[46,47]

Anthelmintic activity: Preliminary anthelmintic activity was tested using various extracts of the CF plant on adult earthworms (*Pheretima posthuma*), followed by tape worms (*Taenia solium*), at doses of 25, 50, 100, and 150 mg/mL and compared with acontrol (0.9% normal saline) and albendazole (25 mg/mL and 50 mg/mL) as a standard. The results revealed that the combined aqueous and methanol extract of CF fruit pulp showed significant anthelmintic activity (26.01 min for the death of the worms) compared with the others with respect to paralysis followed by death for earth worms at 150 mg/mL concentration, and the same combined extract produced death in tape worms at 37.32 min, which was near to the standard drug (28.11 min) (Table 10). Table 10 reveals that albendazole at 50 mg/mL concentration showed 18.30 and 19.20 min for paralysis and 25.20 and 28.11 min for the death of the earth worms and tape worms, respectively.

A literature review reported that tannins and phenolics were known to interfere with energy generation in parasites with the mechanism of uncoupled oxidative phosphorylation,[46] and causes death by binding with free proteins in the gastrointestinal tract of the host animal or glycoprotein on the cuticle of the parasite. The estimation of alkaloids, phenolics, and flavonoids in CF fruit extracts resulted with a high content in the combined methanol and aqueous extract, which supported the strong anthelmintic activity.[31] The result was reported similarly in earlier scientific research.[48,49] In the present study, earthworms were selected for the preliminary study because they are more sensitive than tape worms and round worms.[50] Earlier literature established the anthelmintic activity of *Coscinium fenestratum* stem aqueous extract against round worms and earthworms[51] and death resulted after more than 63 min, whereas in our study, the fruit pulp extract produced death at around 37.32 min, less than stem extract. The result must be due to the effect of the solvent used in extraction.

CONCLUSION

The present investigation has shown that CF fruit pulp has therapeutic activities due to the presence of secondary metabolites and has significant anthelmintic activity due to estimated total alkaloid, phenol, and flavonoid content. The solvent system played a vital role for the activities in which combined aqueous and methanol extract showed the most significant antioxidant and anthelmintic activity as compared with individual methanol and aqueous extracts. This is the first report on CF fruit pulp extract, which may be further explored for its phytochemical profile to recognize the active constituent responsible for anthelmintic activity.

ACKNOWLEDGEMENTS

Authors are thankful to Rajiv Gandhi University of Health Sciences, Bangalore, India for financial assistance of Rs. 1.5 Lakhs as research grant (Principal Investigator) for carry out the present investigation (Order No. RGU: Adv. Res.: Proposal-P-171: 2015-16 DATE: 06-01-2016).

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):166-170
DOI: 10.4274/tjps.49469

ORIGINAL ARTICLE

Protective Effects of Ursolic Acid in the Kidneys of Diabetic Rats

Diyabetik Sıçanların Böbreklerinde Ursolik Asidin Koruyucu Etkileri

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ABSTRACT

Objectives: Diabetes, a heteregenous metabolic and chronic disease, is a growing health problem in most countries. It has been claimed that diabetes is associated with the increased formation of free radicals and decreased in antioxidant potential. Oxidative stress formed in diabetes may cause DNA damage in the tissues. Ursolic acid, a well-known pentacylic triterpene, is commonly used in traditional Chinese medicine due to its beneficial health effects such as antioxidant, anticancer, and antiulcer properties. The aim of this study was to investigate the effects of ursolic acid in the kidneys of Wistar albino rats with streptozotocin-induced diabetes.

Materials and Methods: DNA damage was evaluated in the kidney cells of rats using alkaline comet assays. Oxidative stress parameters such as CAT, SOD, GR, and GSH-Px enzyme activities and total GSH and MDA levels were also evaluated.

Results: Ursolic acid treatment was found to significantly decrease DNA damage, GR enzyme activities, and MDA levels, and significantly increase GSH levels and CAT, SOD and GSH-Px enzyme activities in diabetic rats.

Conclusion: According to our results, it seems that ursolic acid may be beneficial against diabetes-induced renal damage.

Key words: Diabetes, DNA damage, oxidative stress, kidney

ÖZ

Amaç: Metabolik ve kronik bir hastalık olan diyabetin birçok ülkede önemli sağlık sorunu olma durumu artış göstermektedir. Diyabetin artmış serbest radikallerin üretimi ve azalmış antioksidan kapasiteyle ilişkili olduğu iddia edilmektedir. Diyabette oluşan oksiatif stres dokularda DNA hasarına neden olabilir. Antioksidan, antikanser, antiülser özellikler gibi sağlığa yararlı etkileri nedeniyle geleneksel Çin halk tıbbında yaygın olarak kullanılan ursolik asit çok iyi tanımlanmış pentasiklik triterpendir. Bu çalışmanın amacı, streptozotosin ile diyabet oluşturulmuş Wistar albino sıçanların böbreklerine ursolik asidin etkisini değerlendirmektir.

Gereç ve Yöntemler: Bu amaçla, böbrek hücrelerindeki DNA hasarı alkali comet yöntemiyle araştırılmıştır. KAT, SOD, GR ve GSH-Px enzim aktiviteleri ve toplam GSH ve MDA düzeyleri gibi oksidatif stres parametreleri de ölçülmüştür.

Bulgular: Ursolik asit tedavisinin, DNA hasarını, GR enzim aktivitelerini ve MDA seviyelerini önemli ölçüde azalttığı ve diyabetik sıçanlarda GSH düzeylerini ve CAT, SOD ve GSH-Px enzim aktivitelerini önemli ölçüde artırdığı bulundu.

Sonuç: Sonuçlarımıza göre, ursolik asidin diyabetin neden olduğu böbrek hasarında yararlı olabileceği görülmüştür.

Anahtar kelimeler: Diyabet, DNA hasarı, oksidatif stres, böbrek

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Received: 23.06.2017, **Accepted:** 19.07.2017

INTRODUCTION

Diabetes mellitus, a heteregenous metabolic and chronic disease, is caused by an absolute or relative lack of insulin. It is a growing health problem in most countries, especially in developing countries.[1] Around 3% of the world's population has diabetes and it is concluded that it may exceed 5.4% by the year 2025.[2] In Turkey, 7.4% of the population has diabetes, and it is also estimated that the number of patients will increase to 9.6% of the population by 2030.[3]

Studies on diabetes therapy have gained interest due to its unwanted effects on human life e.g. changing lifestyles lead to reduced physical activity, and increased obesity.[3] Drugs used in diabetes therapy have higher costs, limited efficacy and/or significant adverse effects.[4,5] As a result of these factors, patients with diabetes often use alternative therapy such as herbal medicines.[6] Epidemiologic studies have associated diets rich in isoflavones with a lower risk of diabetes and diabetes-related complications.[7,8] However, pharmacologic and toxicologic evidence validating the safety and efficacy of these medicinal plants is not readily available.[9]

For experimental diabetes model in animals, streptozotocin (STZ) and alloxan are the most frequently used drugs. According to the administered dose of these agents, syndromes similar to either type 1, type 2 diabetes mellitus or glucose intolerance can be induced. STZ enters the pancreatic β-cell via a glucose transporter 2 and causes alkylation of deoxyribonucleic acid (DNA).[10] The potential problem with STZ is that its toxic effects are not restricted to pancreatic β-cells because it may cause renal injury and oxidative stress.[11,12]

Ursolic acid (3β-hydroxy-12-urs-12-en-28-oic acid), commonly used in traditional Chinese medicine, is a well-known pentacylic triterpene. *Malus pumila*, *Ocimum basilicum*, *Vaccinium* spp., *Vaccinium* macrocarpon, *Olea europaea*, *Origanum vulgare*, *Rosmarinus officinalis*, *Salvia,* and *Thymus* plants are the main sources of ursolic acid.[13] It has various biologic effects including anticancer, antiulcer, antidiabetic properties due to its antioxidant activity. Ursolic acid has been suggested to increase insulin levels with the preservation of pancreatic β-cells and modulate blood glucose level in diabetic mice.[14]

In this paper, oxidative DNA damage in the kidney cells of STZ-induced diabetic rats were evaluated using alkaline comet assays. Oxidative stress parameters such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GSH-Px) activities, and total GSH and malondialdehyde (MDA) levels in the kidney tissues were also measured to investigate the effects of ursolic acid on diabetes-induced oxidative damage.

MATERIALS AND METHODS

The study was approved by Ankara University Animal Ethics Committee (2015-12-138).

Chemicals

The chemicals used in the study were purchased from the following suppliers: normal-melting agarose and low-melting-point agarose from Boehringer Manheim (Mannheim, Germany); sodium chloride, sodium hydroxide, and potassium chloride from Merck Chemicals (Darmstadt, Germany); dimethyl sulfoxide (DMSO), ethidium bromide (EtBr), Triton-X-100, phosphate-buffered saline (PBS) tablets, STZ and ursolic acid from Sigma-Aldrich Chemicals (St Louis, Missouri, USA); ethylendiaminetetraacetic acid disodium salt dihydrate (EDTA-Na2), natrium lauroy lsarcosinate, and Tris from ICN Biomedicals Inc. (Aurora, Ohio, USA), SOD assay kit, CAT assay kit, GR assay kit, GSH-Px assay kit and GSH assay kit from Cayman Chemicals Co. (Ann Arbor, MI, USA), ketamin hydrochloride from Eczacıbaşı (İstanbul, Turkey).

Animals

Wistar albino rats (180-250 g, n=24) were used in all experiments. The animals were housed in plastic cages with stainless steel grid tops. The rats were maintained on a 12-h light-dark cycle, at controlled temperature (23±2°C) and humidity (50%). The animals were fed with standard laboratory chow and allowed to access feed and drinking water ad libitum. The blood glucose levels of the animals were measured before the experiments. The rats were treated humanely according to the Helsinki Declaration of 2008 and with regard for the alleviation of suffering.

STZ-induced diabetes mellitus model

Wistar rats were subjected to type 1 diabetes through STZ injection (60 mg/kg in freshly prepared PBS) as previously described.[15] Two days later, blood was taken from the tails of the rats using a lancet to measure the blood glucose levels with a glucometer (Plusmed). Rats with a blood glucose level higher than 250 mg/dL were considered to be diabetic.

Experimental design

The rats were divided into four groups:

Group 1: Sham group (n=6). This group consisted of animals treated with oral saline alone.

Group 2: Diabetic group (n=6). This group consisted of animals in which only diabetes was induced and the animals were treated with oral saline.

Group 3: Ursolic acid-treated group (n=6). This group consisted of animals treated with a dose of 50 mg/kg b.w. per oral ursolic acid (in saline) for 28 days.

Group 4: Ursolic acid-treated diabetic group (n=6). This group consisted of animals treated with a dose of 50 mg/kg b.w. per oral ursolic acid (in saline) for 28 days following the induction of diabetes.

Ursolic acid dose (50 mg/kg b.w. per oral) was selected according to our unpublished studies. At the end of the experimental period, all animals were decapitated under anesthesia (90 mg/kg ketamine hydrochloride, i.p.). The kidneys were removed. The organs were examined for changes in size, color, and texture. The samples were kept in the dark at 4°C and processed within 4 hours for comet assays. The kidney homogenates were kept at -80°C for the determination of oxidative stress parameters.

Determination of oxidative stress parameters

Oxidative stress parameters were assayed in the plasma samples and in the liver homogenates. The liver tissues were weighed and extracted following the homogenization and sonication procedure.[16]

The determination of CAT, SOD, GR, and GSH-Px enzyme activities and GSH and MDA levels in the kidney tissues were performed spectrophotometrically using CAT, SOD, GR, GSH-Px, GSH, and MDA assay kits (Cayman Chemicals Co., Ann Arbor, MI, USA) at 540, 440, 340, 340, 420, and 535 nm, respectively. The results are expressed as mmol/min/mg for enzyme activities, μM for GSH levels, and nmol/g for MDA levels.

Determination of DNA damage

Kidney homogenates were used for the comet assays. The kidney tissues were carefully dissected from their attachments and totally excised. Preparation of a single-cell suspension from the organs was performed according to standard procedures.[17,18] In brief, approximately 0.2 g of each organ was placed in 1 mL chilled mincing solution [Hanks' balanced salt solution (HBSS) with 20 mM EDTA and 10% DMSO] in a Petri dish and chopped into pieces using a pair of scissors. The pieces were allowed to settle and the supernatant containing the single-cell suspension was taken. The concentrations of renal and hepatic tissue cells in the supernatant were adjusted to approximately 2 x 106 cells/mL in HBSS containing 20 mM EDTA / 10% DMSO.

The alkaline comet assay technique of Singh et al.[19], as further described by Aydın et al.[20] and Bacanlı et al.[21] was followed.

The dried microscopic slides were stained with EtBr (20 μg/mL in distilled water, 60 μL/slide), covered with a cover-glass prior to analysis using a Leica® fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd, Liverpool, UK) to determine the extent of DNA damage after the electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, 100 nuclei per slide were examined at 40x magnification. The results are expressed as percent of DNA in tail ('tail intensity').

Statistical analysis

Statistical analysis was performed using the SPSS for Windows 20.0 computer program. Differences between the means of data were compared using the One-way variance analysis (ANOVA) test, and post-hoc analysis of group differences was performed using the least significant difference test. The Kruskal-Wallis K test followed by the Mann-Whitney U test was used to compare parameters displaying abnormal distribution between the groups. The results are given as mean ± standard deviation. P values of less than 0.05 were considered as statistically significant.

RESULTS

Assessment of oxidative stress parameters

The CAT, SOD, GR and GSH-Px enzyme activities and GSH and MDA levels in the kidney tissues were shown in the Table 1.

CAT, SOD, and GSH-Px enzyme activities and GSH levels were found to be significantly lower in the diabetic group compared with the sham group ($p<0.05$). CAT, SOD, and GSH-Px enzyme activities and GSH levels were significantly increased in the ursolic acid-treated diabetic group compared with the diabetic group ($p<0.05$). There were no significant differences between the sham and ursolic acid-treated diabetic group.

Renal GR enzyme activities and MDA levels were found to significantly increased in the diabetic group compared with the sham group ($p<0.05$). However, the levels were found to significantly decreased in the ursolic acid-treated diabetic group compared with the diabetic group ($p<0.05$). There was no significant difference between the sham and ursolic acid-treated diabetic group in terms of MDA levels ($p<0.05$).

Ursolic acid alone caused no significant changes in all studied oxidative stress parameters compared with the sham group.

Table 1. Oxidative stress parameters in the kidneys of the experimental groups

	Group 1	Group 2	Group 3	Group 4
CAT activity (nmol/min/mL)	15.130±2.189[b]	10.463±4.711[a]	167.390±8.200[b]	136.386±29.559[a,b]
SOD activity (U/mL)	0.451±0.304[b]	0.241±0.147[a]	0.921±0.348[b]	0.738±0.129[b]
GSH-Px activity (nmol/min/mL)	124.126±2.563[b]	56.514±5.466[a]	138.145±5.795[b]	125.657±7.120[b]
GR activity (nmol/min/mL)	3.737±1.281[b]	11.382±6.265[a]	4.976±1.239[b]	5.956±0.423[b]
GSH levels (μM)	9.938±3.628[b]	5.419±0.584[a]	8.396±2.125[b]	7.965±1.458[b]
MDA levels (nmol/g)	13.452±5.128[b]	24.367±4.785[a]	14.108±3.025[b]	15.203±7.520[b]

The values are expressed as mean ± standard deviation; a: Statistically different from the sham group ($p<0.05$), [b]: Statistically different from the diabetes group ($p<0.05$).
Group 1: Sham, Group 2: Diabetic rats, Group 3: Ursolic acid (50 mg/kg)-treated rats, Group 4: Ursolic acid (50 mg/kg)-treated diabetic rats.
CAT: Catalase, SOD: Superoxide dismutase, GSH-Px: Glutathione peroxidase, GR: Glutathione reductase, GSH: Glutathione, MDA: Malondialdehyde

Assessment of DNA damage

The DNA damage expressed as tail intensity in the kidney cells of rats in comet assays is shown in the Figure 1.

In all samples studied, there were no statistically significant differences in tail intensity between the sham group and the ursolic acid-treated groups ($p>0.05$). The DNA damage was found significantly higher in the diabetic group compared with the sham group ($p<0.05$). Ursolic acid treatment in the diabetic group was found to decrease the DNA damage significantly ($p<0.05$).

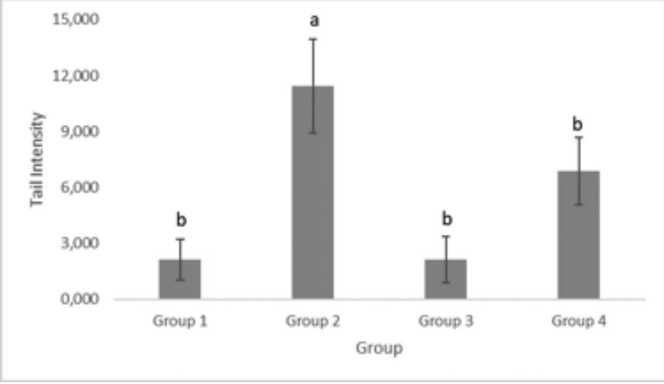

Figure 1. DNA damage of the experimental groups of the rats

The values are expressed as mean ± standard deviation a: Statistically different from the sham group ($p<0.05$); b: Statistically different from the diabetic group ($p<0.05$)

Group 1: Sham, Group 2: Diabetic rats, Group 3: Ursolic acid (50 mg/kg)-treated rats, Group 4: Ursolic acid (50 mg/kg)-treated diabetic rats

DISCUSSION

In the present study, we evaluated the protective effects of ursolic acid against the renal effects of STZ-induced diabetes in rats. STZ is a pancreatic β-cell toxin that causes irreversible necrosis of β-cells.[16] When injected into animals at different doses, it causes mild-to-severe types of diabetes according to the dose used.[22]

Multiple factors can cause oxidative stress in diabetes. The most important factor is glucose autoxidation, which leads to the production of free radicals. Other factors include cellular oxidation/reduction imbalances and reduction in antioxidant defenses including decreased cellular antioxidant levels and a reduction in the activity of enzymes that dispose of free radicals.[23]

It has been shown that oxidative stress exists in patients with diabetes, as evidenced by increased total antioxidant capacity in the saliva and blood of patients.[24] MDA has been identified to bind and damage DNA. It is a direct product of lipid peroxidation. In different studies, it has been demonstrated that MDA levels were significantly increased in different tissues such as liver and kidney tissues.[25,26] Increased MDA levels were shown in patients with diabetes[27] and diabetic rats.[28]

Our findings are consistent with the data of some recent studies that showed changes in oxidative stress parameters in diabetes. In the present study, we observed that GR enzyme activities and MDA levels were significantly increased, and GSH levels and CAT, SOD, and GSH-Px enzyme activities were significantly decreased in diabetic rats.

Recently, there has been a growing interest in replacing synthetic diabetic drugs with natural antioxidants from plant materials.[29] Phytochemicals with antioxidant effects include cinnamic acids, coumarins, diterpenes, flavonoids, lignans, monoterpenes, phenylpropanoids, tannins, and triterpenes.[30] Wang et al.[31] demonstrated that oleanolic acid (0.1 and 0.2%) and ursolic acid (0.1 and 0.2%) markedly suppressed renal aldose reductase activity and enhanced glyoxalase I activity, which contributed to decrease renal advanced glycation end products formation and improvedd renal functions. The impact of these two triterpenes on mRNA expression of renal aldose reductase and glyoxalase I revealed that the effects of these agents occurred at the transcription level. Low-dose ursolic acid (0.01% in food) administration in STZ-induced diabetic mice for three months, glomerular hypertrophy and type IV collagen accumulation in the kidneys were found to be markedly ameliorated.[32] In a 16-week study on rats with STZ-induced diabetes, ursolic acid treatment prevented biochemical and histopathologic changes in the kidneys associated with diabetes such as alterations in renal function and increases in oxidative stress, NF-κB activity, and P-selectin expression in the kidneys.[33] In our study, we found that ursolic acid regulated all of the alterations of oxidative stress parameters in diabetes. Ursolic acid treatment was found to significantly decrease GR enzyme activities and MDA levels and significantly increase GSH levels and CAT, SOD, and GSH-Px enzyme activities in diabetic rats.

Kushwaha et al.[34] determined the DNA damage in lung, liver, aorta, heart, kidney, pancreas, and blood samples of experimentally-induced diabetic rats using comet and endonuclease III and formamidopyrimidine (fpg) modified comet assays. The authors found that DNA damage was significantly greater in the diabetic group compared with the non-diabetic group. In our previous study with ursolic acid, we reported the antigenotoxic effect of this phytochemical against hydrogen peroxide induced oxidative DNA damage in the human lymphocytes and Chinese hamster fibroblast cells.[35] In the present study, DNA damage in diabetic rats was found to be greater compared with the sham group. Ursolic acid treatment was found to significantly decrease oxidative DNA damage in diabetic rats.

CONCLUSIONS

Our results are in accordance with previous reports about diabetes, its complications and the effects of natural antioxidants against diabetes. We conclude that ursolic acid treatment may be preventive against diabetes and its effects. Nevertheless, further *in vitro* and *in vivo* studies are needed to elucidate their efficacy, mechanism, and toxicity on diabetes treatment.

ACKNOWLEDGEMENTS

This work was supported by the Scientific and Technological Research Council of Turkey (Grant number: 114S919).

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):171-177
DOI: 10.4274/tjps.91885

ORIGINAL ARTICLE

Development and Pre-clinical Study of Anti-Allergic Cream Containing Dexamethasone and Chlorpheniramine

Deksametazon ve Klorfeniramin İçeren Anti-alerjik Kremlerin Geliştirilmesi ve Klinik Öncesi Çalışması

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ABSTRACT

Objectives: In this study, we aimed to develop and optimize an anti-allergic cream containing dexamethasone and chlorpheniramine using the design of experiments (DoE) method. The optimized product was investigated for its physicochemical properties and *in vivo* therapeutic effects in rabbits.

Materials and Methods: The creams were formulated using the simple mixing process, which was optimized by the Design Expert software. The products were then evaluated the properties such as pH, skin diffusion profile, short-term stability, qualitative, and assay, using the newly validated UV-Vis spectrophotoscopy quantitative method. *In vivo* efficacy tests in rabbits of the best products were compared with the marketed Phenergan® (promethazine 2%).

Results: The UV-Vis method used to simultaneously determine the amount of both dexamethasone and chlorpheniramine was successfully developed and validated. Using the DoE method, it was clear that the release profile of dexamethasone depended on the amount of sodium lauryl sulfate, propylene glycol, and DMSO. In contrast, only DMSO affected the release pattern of chlorpheniramine. The best formulation was optimized by the software. The product showed acceptable parameters in pH (5.7±0.1), short-term stability over 10 days, and skin diffusion profiles of 20.47±1.25% and 4.92±0.42% after 40 min for dexamethasone and chlorpheniramine, respectively. In addition, the product demonstrated no observable inflammatory response in the experimental animals. Also, it illustrated 2-fold better anti-allergic efficacy than the marketed product (i.e., 27.2 compared with 43.4 min in the recovery time).

Conclusion: We were successful in developing and optimizing an anti-allergic cream containing dexamethasone and chlorpheniramine. The best product satisfied all required parameters. Interestingly, our product showed higher efficacy than Phenergan®. These results can be a background for further clinical trials.

Key words: Dexamethasone, chlorpheniramine, allergic, design of experiments, *in vivo* study

ÖZ

Amaç: Bu çalışmada deney tasarımı (DoE) yöntemi kullanılarak deksametazon ve klorfeniramin içeren anti-alerjik kremlerin geliştirilmesi ve optimize edilmesi amaçlandı. Optimize edilen ürünün, tavşanlarda fizikokimyasal özellikleri ve *in vivo* terapötik etkileri araştırıldı.

Gereç ve Yöntemler: Kremler, Design Expert yazılımı tarafından optimize edilen basit karıştırma işlemi ile formüle edildi. Daha sonra pH, deri difüzyon profili, kısa süreli stabilite, kalitatif tayin ve yeni valide edilmiş UV-Vis spektrofotoskopi yöntemi kullanılarak ürünlerin kantitatif miktar tayini gibi özellikleri değerlendirildi. En iyi ürünlerin tavşanlarda *in vivo* etkinlik testi, piyasadaki Phenergan® (prometazin %2) ile karşılaştırıldı.

Bulgular: Hem deksametazon hem de klorfeniramin miktarını eşzamanlı olarak belirlemek için UV-Vis yöntemi başarıyla geliştirildi ve valide edildi. DoE yöntemini kullanarak, deksametazonun salım profilinin, sodyum lauril sülfat, propilen glikol ve DMSO miktarına bağlı olduğu açıktı. Oysa klorfeniramin salınım modelini sadece DMSO etkiliyordu. Ayrıca, en iyi formülasyon yazılım tarafından optimize edildi. Ürün, deksametazon ve klorfeniramin için sırasıyla pH'de (5.7±0.1), 10 gün içinde kısa süreli stabilite, 40.4 dakika sonra %20.47±1.25 ve %4.92±0.42'lik deri difüzyon profilleri için kabul edilebilir parametreler gösterdi. Buna ek olarak, ürün, deney hayvanlarında gözlemlenebilir bir enflamatuvar tepki göstermedi. Ayrıca, satılan ürüne kıyasla 2 kat daha iyi anti-alerjik etkinlik (yani, geri kazanım süresinde 43.4 dakika ile karşılaştırıldığında 27.2 dakika) gösterdi.

Sonuç: Deksametazon ve klorfeniramin içeren anti-alerjik kremin geliştirilmesi ve optimize edilmesi konusunda başarılı olduk. En iyi ürün gerekli tüm parametreleri sağlamıştır. İlginç bir şekilde, ürünümüz Phenergan®'dan daha yüksek etkililik gösterdi. Bu sonuçlar, klinik araştırmalarda ileri çalışmalar için bir zemin olabilir.

Anahtar kelimeler: Deksametazon, klorfeniramin, alerjik, deney tasarımı, *in vivo* çalışma

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Received: 23.06.2017, Accepted: 02.08.2017

INTRODUCTION

Allergy, a common disorder caused by an exposure to allergens, followed by an immune system response, can be classified into many types such as allergic rhinitis (i.e., respiratory system), asthma, drug allergy, food allergy, general allergy (i.e., pollens as allergens), insect allergy, and skin allergy. Among them, skin allergy is one of the most common types, especially in children. In 2010, in the United States of America, 10% of Asian children, 12% of white children, and 17% of African American children had skin allergies.[1] Moreover, in 2012, the lifetime incidence of urticaria (i.e., hives, allergic rash) worldwide was in excess of 20% in humans.[2]

The general treatments for skin allergy include anti-histamines (i.e., chlorpheniramine), glucocorticoids (i.e., dexamethasone), epinephrine (adrenaline), mast cell stabilizers (i.e., cromolyn), as well as anti-leukotriene agents (i.e., montelukast). The standard oral route of these medications encounters many unwanted averse effects. For example, the use of chlorpheniramine could lead to constipation, dizziness, headache, nausea, loss of appetite, or rarely dyskinesias, tremors, tachycardia, diplopia, dysuria, and even fatal agranulocytosis.[3-5] Hence, a suitable route (i.e., local administration) should be developed. Cream formulations have been investigated intensively in recent years for skin application. Their excellent skin compatibility, high stability in normal preservative conditions, as well as ease of processability, make creams become the 'formulation of choice' for scientists.

The idea of drug combination has been proposed recently in order to reduce the dose of the individual therapeutic agents and increase the efficacy due to the synergism between the active substances.[6-8] In allergy treatment, the combination of one anti-histamine, namely chlorpheniramine, and one glucocorticoid, such as dexamethasone, is often used. However, the fact that limited skin cream products containing both of these pharmaceutic agents are available in the market is undeniable. To the best of our knowledge, only one combination, namely Dexalergin®, manufactured by IVAX (Argentina) is present on the market. Also, it is worth noticing that Dexalergin® has neomycin sulfate, an antibacterial agent, along with chlorpheniramine and dexamethasone (http://www.medicatione.com/?c=drug&s=dexalergin%20cream). Hence, no 'pure' combination of these two drugs is available.

Chlorpheniramine or chlorphenamine (Figure 1A), commonly marketed in the form of chlorpheniramine maleate, is the first generation of alkylamine compounds for anti-histamine purposes. It also possesses anti-depressant and anti-anxiety properties, although not generally approved.[9,10] Chlorpheniramine's primary mechanism of action is as a histamine receptor H_1 competitive antagonist, which consequently hinders the allergic response caused by histamine. On the other hand, dexamethasone (Figure 1B) is a steroid compound, which can inhibit the formation of inflammatory and allergic mediators such as histamine, prostaglandins, as well as leukotrienes.

With all these reasons taken together, we came up with the idea of the development and pre-clinical study of an anti-allergic cream containing dexamethasone and chlorpheniramine. The research was conducted using the first step of experimental design and optimization to select the best formulation, followed by quantitative method development and validation, characterization of the formulas (i.e., pH, skin diffusion profile, short-term stability, qualitative, and assay), and *in vivo* efficacy test in rabbits.

MATERIALS AND METHODS

Materials

Standard chlorpheniramine and dexamethasone were bought from the Institute of Drug Quality Control, Ho Chi Minh City, lot numbers QT021050809 and QT013060909, with purities of 99.32% and 99.43%, respectively. The chlorpheniramine and dexamethasone ingredients were imported from India and China, lot numbers 1010149 and 100505, purity 98.7% and 99%, respectively. Cetylstearyl alcohol, lot number 10099, was bought from Singapore; sodium lauryl sulfate, lot number 23263, was purchased from Indonesia. Dimethyl sulfoxide (DMSO), propylene glycol, glycerol, liquid paraffin, methanol, benzene, ethanol, chloroform, and hydrochloric acid were imported from China, all were of pharmaceutical grades. Phenergan® cream

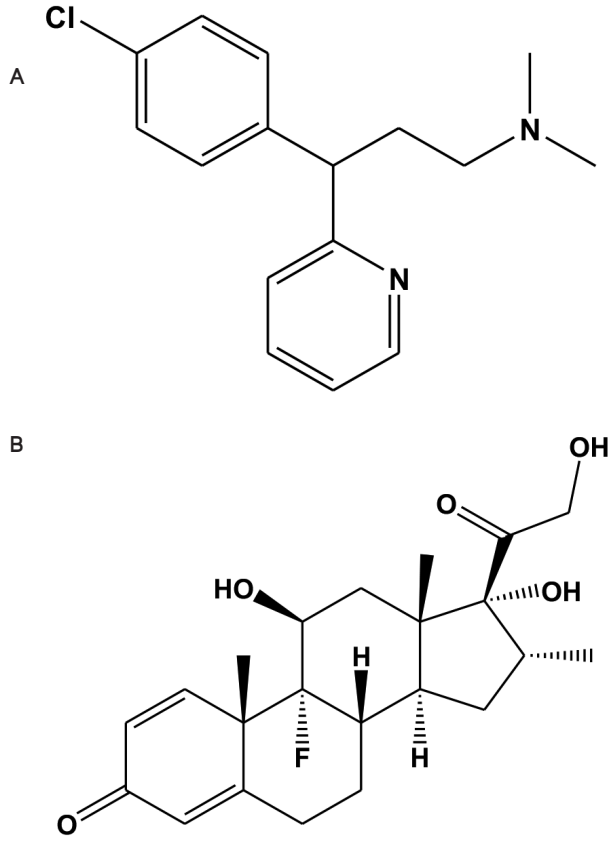

Figure 1. A) Chlorpheniramine, $C_{16}H_{19}ClN_2$, MW: 274.79, 3-(4-chlorophenyl)-N,N-dimethyl-3-(pyridin-2-yl) propan-1-amine. B) Dexamethasone, $C_{22}H_{29}FO_5$, MW: 392.46, (8S, 9R, 10S, 11S, 13S, 14S, 16R, 17R)-9-fluoro-7,8,11,12,13,15,16,17-octahydro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6H-cyclopenta[a]phenanthren-3 (9H,10H,14H)-one

(promethazine 2%, Sanofi-Aventis) was bought from a Ngoc Anh drugstore, Can Tho, Vietnam. Mature rabbits were supplied by the Department of Pharmacology, Can Tho University of Medicine and Pharmacy. Ethical approval was granted by the Ethics Committee (No: CTU 2016-5-017), based on the Animal Care and Use Committee guideline of the same university.

Drug quantitation

To determine the amount of drug either in the formulations or the release medium, we developed and validated a ultraviolet/visible spectrometry (UV-Vis) spectrophotoscopic method that could measure both chlorpheniramine and dexamethasone under the same conditions. The samples were dissolved in methanol and were measured at the wavelengths of 239 nm and 262 nm for dexamethasone and chlorpheniramine, respectively. All validation values, including specificity, linearity, precision, and accuracy were determined using a UV-Vis spectrophotometer (Hitachi U2800, Japan).

Formulation

The formulations were produced following a simple mixing process. The oil phase, which was composed of cetylstearyl alcohol and liquid paraffin, was heated to approximately 70°C. Then, it was mixed with the water phase, which comprised water, glycerol, sodium lauryl sulfate at the same temperature, using a high-speed homogenizer (Ultra Turrax T-25, IKA, Germany) to make the cream base. Chlorpheniramine in water and dexamethasone in propylene glycol and DMSO were then added and mixed with the cream base. The final product had the concentrations of chlorpheniramine and dexamethasone of 1% w/w and 0.1% w/w, respectively.

Experimental design

The Design-Expert software (version 10.0, Stat-Ease, Inc., Minnesota, U.S.A.) was used to design and optimize the formulation. The response surface methodology with the linear function model was chosen for the development part. Three independent factors, including the amount (g) of sodium lauryl sulfate (X_1), propylene glycol (X_2), and DMSO (X_3). The concentrations of two active compounds as well as other excipients such as cetylstearyl alcohol, liquid paraffin, and glycerol, were kept constant. Three factors were studied at five different levels ($-\alpha$, -1, 0, +1, $+\alpha$) using a central composite design. The α value of 1.68 was chosen to maintain the rotatability and orthogonality of the design. Two response variables were clarified, namely the percentage of *in vitro* release of dexamethasone (Y_1) and chlorpheniramine (Y_2) through the rabbit skin after 40 min. The predicted function can be defined as follows:

$$Y=b_0+b_1 X_1+b_2 X_2+b_3 X_3$$

where Y is the predicted response, X_1, X_2, X_3 are the independent factors, b_0 is the intercept, and b_1, b_2, b_3 represent linear coefficients.

Analysis of variance (ANOVA) was used to calculate the significance of the model, and p values of less than 0.05 were considered significant. The optimal formulation was also predicted using the software.

Characterizations

Physical characteristics

The appearance of the final product was evaluated by the naked eye. The suitable formulation must possess a white soft creamy texture with no observable separation between the oil and the water phases. The formulation pH was determined as follows: weigh 5 g cream and mix with 50 mL of distilled water for 5 min, and measure the pH (Mettler Toledo, Switzerland) of the filtrate of the mixture after filtration. The acceptable pH is in the range of 5.5 to 6.

Short-term stability

To test the stability of the formulation, an acceleration study was conducted. The final product was kept at 40°C for ten days, with 2 hours of 50°C incubation each day. The criteria included the appearance, texture, color, and smell.

Skin diffusion profile

The end points of skin diffusion tests were evaluated using the Franz cell method. In brief, 200 mg of the final product was weighed and applied to the rabbit skin with an area of 3.14 cm^2 in the donor chamber. The acceptor chamber was filled with 18 mL of methanol and stirred with magnetic bars. The system was maintained at 37°C using a water bath. After 40 min, the amounts of chlorpheniramine and dexamethasone released into the acceptor chamber were measured by validated using UV-Vis spectrophotoscopy.

Drug identification

Thin layer chromatography (TLC) was used to identify both drugs (i.e., chlorpheniramine, dexamethasone) in the cream products. The reference (i.e., standard drugs) and the samples were dissolved and extracted, respectively, in methanol for 60 min. Ten microliters of the two samples, including the reference and the test, were applied onto the chromatography layers (Silica gel GF254, Merck, Inc., U.S.A). The mobile phase was composed of benzene - ethanol - NH_4OH (85:15:1 v/v/v). The best components and ratio of the mobile phase were preliminarily investigated based on the polarity of both compounds. The spots were visualized under UV light at the wavelength of 254 nm.

Assay

To determine the amount of drugs in the cream product, 1 g of the cream (equivalent to 10 mg of chlorpheniramine and 1 mg of dexamethasone) was weighed, extracted with methanol for 60 min, and filtered through 0.22-µm millipore filters (Merck, Inc., U.S.A.). The samples were then underwent UV spectrophotoscopy measurements using a validated method with methanol as a blank sample. The percentage of drugs was calculated as follows:

$$\% \text{ Dexamethasone} = \frac{C_1 x 434.5 x 10^3}{1 x 99\%} \quad x100$$

$$\% \text{ Chlorpheniramine} = \frac{C_2 x 390.87 x 10^3}{10 x 98.7\%} \quad x100$$

where C_1 and C_2 are the concentrations of dexamethasone and chlorpheniramine, respectively.

In vivo tests

Inflammatory response

Inflammatory responses of the cream products were evaluated using rabbits. Mature rabbits (weight ≥2 kg) were cultivated in normal conditions 5 days prior testing. For the duration of 4 hours, the cream samples (0.5 g) and the control (i.e., cream base) were applied onto hair-free areas on the rabbits backs, with an area of approximately 10 cm x 15 cm, at 25±3°C, 30-70% humidity, and light-dark intervals of 12 hours. The remaining creams were then washed off, and skin inflammation was observed at 1, 24, 48, and 72 hours afterwards. A score, ranging from 0-0.5 for no significant inflammatory response, to 3.5-4.0 for serious inflammatory response (i.e., redness, swelling, animal pain) was given for each corresponding reaction, and compared with the control and the blank area (i.e., no treatment). The experiment was performed in triplicate, and the degrees of inflammatory reaction of the product were recorded and averaged.

Efficacy test

The efficacy test of the cream, in comparison with the marketed Phenergan® (promethazine 2%) was performed similarly to the inflammatory response test. The chloroform-induced allergy model was used. One milliliter of chloroform was applied, using soft tissues, onto the hair-free areas (6 squares, 2.5 cm x 2.5 cm) for 40 seconds. Then, samples including (a) negative control; (b) cream, 0.05 g; (c) cream, 0.1 g; (d) positive control; (e) Phenergan®, 0.05 g; (f) Phenergan®, 0.1 g, were applied (Figure 2). The efficacy test ran for one hour and the time until the rabbit skin returned to normal (i.e., the recovery time) was compared with the negative control and recorded. The experiment was repeated ten times, with ten different rabbits, and the time average values were reported. Student's t-test was used to compare the significant differences between our products and the marketed Phenergan®.

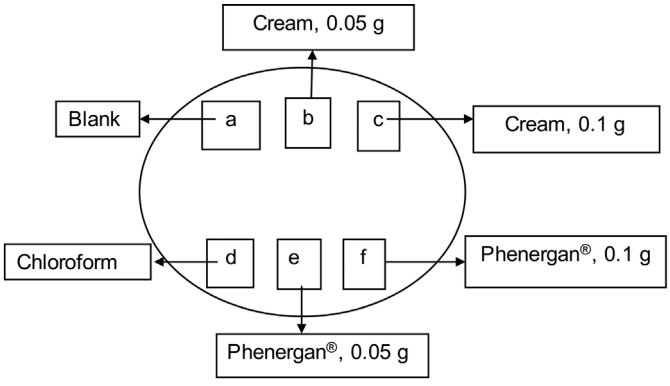

Figure 2. Diagram of drug applicable areas on the rabbit hair-free skin

RESULTS AND DISCUSSION

Drug quantitation

The concentrations of both active substances were measured simultaneously in the same samples using two different wavelengths, 239 nm for dexamethasone and 262 nm for chlorpheniramine. Due to the fact that these two substances have absorbance values at both wavelengths, and the range of the two wavelengths is more than 10 nm (i.e., 23 nm), we calculated the concentrations based on the following equations:

$$A_{(\lambda_1)} = \varepsilon^1_{\lambda_1} C_1 + \varepsilon^2_{\lambda_1} C_2$$

$$A_{(\lambda_2)} = \varepsilon^1_{\lambda_2} C_1 + \varepsilon^2_{\lambda_2} C_2$$

where A_1 and A_2 are the absorbance values at 239 nm and 262 nm; $\varepsilon^1_{\lambda_2}, \varepsilon^2_{\lambda_1}, \varepsilon^1_{\lambda_2}, \varepsilon^2_{\lambda_2}$ are the molar absorptivity of the chlorpheniramine and dexamethasone at 239 nm and 262 nm, respectively; and C_1 and C_2 are the concentrations of chlorpheniramine and dexamethasone.

By calculating from the standard samples, the $\varepsilon^1_{\lambda_2}, \varepsilon^2_{\lambda_1}, \varepsilon^1_{\lambda_2}, \varepsilon^2_{\lambda_2}$ values were determined as 4336.0, 16260.8, 5351.6, and 8608.7, respectively.

The specificity was confirmed by the perfect overlay of the standard and the test samples spectra (data not shown). Moreover, the ratio of absorbance values between the blank (i.e., methanol) and the test samples was less than 1% (i.e., 0.57% at 239 nm and 0.51% at 262 nm), indicating the sensitivity of the method.

The linearity of the method was also specified (Figure 3). These two substances showed linearity in the range of 0-70 ppm for chlorpheniramine and 0-7 ppm for dexamethasone with coefficients of determination (R^2) at their corresponding maximal absorption wavelengths of 0.9993 and 0.9981, respectively. The linearity of these two was also seen in the other wavelength (i.e., 239 nm for chlorpheniramine) (data not shown).

In the precision test, six independent samples containing both chlorpheniramine and dexamethasone were prepared and measured. The relative standard deviations (SDs) of 0.37% for chlorpheniramine and 1.59% for dexamethasone, which is less than 2%, indicated the precision of the method. In addition, the

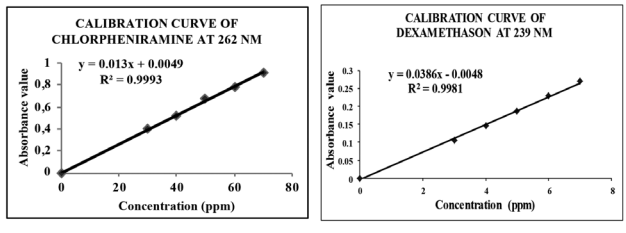

Figure 3. Calibration curves of chlorpheniramine and dexamethasone at their maximal absorption wavelengths

recovery values of these two substances at the concentrations of 80%, 100%, and 120% compared with the standard ones, were in the range of 98-102%, which demonstrated the method accuracy. In summary, the quantitative method was validated and satisfied all required parameters.

Experimental design

From the Design-Expert software, a total of 20 formulations were proposed, formulated, and evaluated in terms of *in vitro* release (%) of dexamethasone (Y_1) and chlorpheniramine (Y_2) through the rat skin after 40 min. The optimal correlations are shown as follows:

$$Y_1 = 3.9325 + 0.3375X_1 + 0.5025X_2 + 0.71X_3 \quad (1)$$

$$Y_2 = 3.9325 + 0.30125X_3 \quad (2)$$

where X_1, X_2, X_3 are the amount (g) of sodium lauryl sulfate, propylene glycol, and DMSO, respectively.

The regression model was tested using ANOVA. The high F values of 215.05 and 145.63 for equation (1) and (2), respectively, as well as the low p values of less than 0.0001 for both equations, indicate the statistical significance of the model. Moreover, the insignificances of the lack of fit values with p=0.473 and p=0.359 clearly demonstrate that the model fitted well. These confirm the reliability of this model.

From the equations, it is likely that the fact that the release profile of dexamethasone (Y_1) was positive linearly depended on the amount of sodium lauryl sulfate, propylene glycol, and DMSO. In contrast, only DMSO affected the release pattern of chlorpheniramine (Y_2) in a similar manner. This can be explained by the extremely low solubility of dexamethasone in water (0.1 mg/mL), which led to the help of surfactant (i.e., sodium lauryl sulfate) and organic solvents in its dissolution and penetration through the rabbit skin.[11] On the other hand, chlorpheniramine, which has a water solubility at 160 mg/mL, 1600 times higher than that of dexamethasone, needed only DMSO to pass through the same skin (http://www.hmdb.ca/metabolites/HMDB01944).

Optimal conditions were determined based on the correlations between these factors, with the desired limits as to maximize the percentage of drug penetrated through the rat skin after 40 min, as well as to keep the concentrations of surfactant and organic solvents at acceptable values. Based on the literature, the concentrations of sodium lauryl sulfate, propylene glycol, and DMSO in the skin cream should be less than 1%, 30%, and 50%, respectively, in order to prevent dermatitis and other kinds of skin damage.[12-16] The optimal formulation was then prepared and further studied.

Characterizations

Physical characteristics

The appearance of the optimized product possessed a white soft creamy texture with no observable separation between the oil and the water phases. The formulation pH was 5.7±0.1, which was within the acceptable pH range of 5.5 to 6.

Short-term stability

The acceleration test was conducted with the final products over 10 days. After this duration, all formulations exhibited excellent stability without any significant changes in term of appearance, texture, color, and smell.

Skin diffusion profile

The Franz cells method was used to evaluate the diffusion profile of the product. Rabbit skin was used due to its reproducibility property. The optimal release profiles of dexamethasone and chlorpheniramine were 20.47±1.25% and 4.92±0.42% (mean ± SD, n=3), respectively, after 40 min of application. The relationship between the formulation ingredients and the skin diffusion (i.e., release) profiles of these two substances were optimized in the experimental design part.

Drug identification

TLC pictures of the two active substances, using benzene - ethanol - NH_4OH (85:15:1 v/v/v) as a mobile phase, are illustrated in Figure 4. Clearly, the optimal cream product demonstrates a suitable separation of dexamethasone and chlorpheniramine, with no observable interference.

Assay

Following the procedure in the method section, the concentrations of dexamethasone and chlorpheniramine in the optimal product were 100.6±0.84% and 99.9±0.37% (mean ± SD, n=3), respectively. These results indicated that the final product satisfied the quantitative requirement for both substances.

In vivo tests

Inflammatory response

No significant inflammatory response was encountered under the experimental conditions stated in the method section. The final product demonstrated good compatibility with the rabbit skin, without any redness, swelling, or observable reactions, compared with the control groups. The average score for three

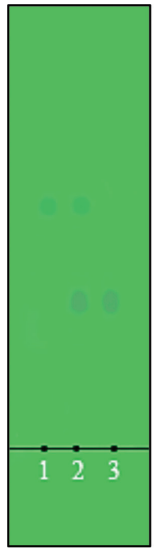

Figure 4. Thin layer chromatogram of the final cream product. (1) Standard dexamethasone, (2) Test sample, (3) Standard chlorpheniramine. Mobile phase: benzene – ethanol – NH_4OH (85:15:1 v/v/v). Dots were visualized under ultraviolet 254 nm

independent tests was 0, which is in the range of 'no significant inflammatory response' (i.e., 0-0.5). Hence, we could conclude that our product showe no irritation on the skin.

Efficacy test

Preliminary studies for the induction dose and time of chloroform to obtain an allergic response were conducted. We found that 1 mL of chloroform in 40 seconds was the best option. The same dose at 60 seconds could induce irreversible ulceration, which is not a good choice for an anti-allergic study. Moreover, the effects of chloroform were insignificantly different amongst the rabbit skin areas (Figure 5).

The efficacy of our cream, in comparison with the marketed Phenergan® (promethazine 2%) in reducing the allergic response in rabbits was evaluated, in terms of recovery time (i.e., the necessary time required for the rabbit skin to recover after chloroform exposure). The results are demonstrated in Figure 6. In brief, similar to Phenergan®, our cream was a dose-dependent product, with a significant 2-fold shorter recovery time in the 0.1 g dose (27.2±1.42 min) compared with the 0.05 g dose (55.8±1.54 min) ($p<0.05$, n=10). Interestingly, our optimal product showed meaningfully better therapeutic effects (i.e., lower recovery time) than the marketed product Phenergan® at both doses ($p<0.05$, n=10). This could be due to the synergistic effect of dexamethasone and chlorpheniramine. Finally, the use of a combination product (i.e., more than one active substance in a product) might reduce the dose of each individual substance, and hence, hinder unwanted effects.

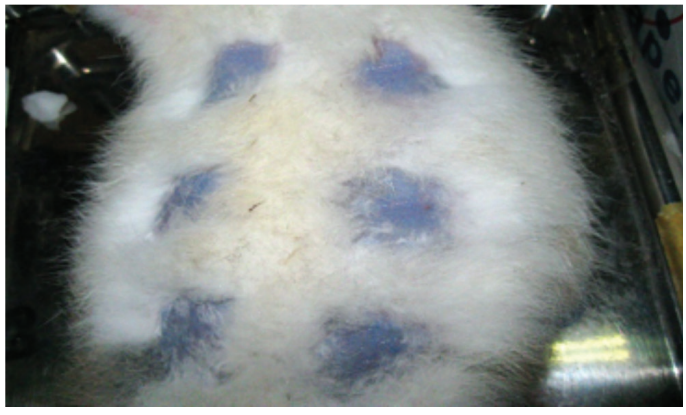

Figure 5. Chloroform-induced allergy model. One milliliter of chloroform solution for 40 seconds could induce the allergic response similarly amongst the rabbit skin areas

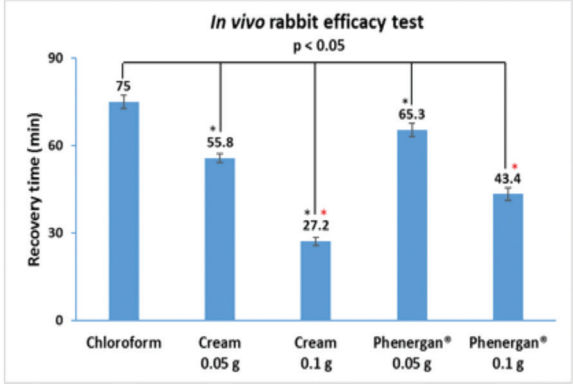

Figure 6. *In vivo* rabbit efficacy test, in the comparison between the optimal cream and the marketed product Phenergan®. The results are demonstrated as mean ± standard deviation, n=10. The black line indicates the significance ($p<0.05$) between the negative control chloroform and the formulations. The black and red stars show the differences between the doses and the formulations ($p<0.05$)

CONCLUSION

In summary, anti-allergic cream containing dexamethasone and chlorpheniramine was successfully prepared, optimized, characterized, as well as evaluated in *in vitro* and *in vivo* test. The optimal formulation was considered a novel formula, which balanced the efficacy and toxicity of the product, regarding organic solvents such as propylene glycol and DMSO. Additionally, our product satisfied all of the evaluation parameters (i.e., physical properties, stability, skin diffusion profiles, drug identification, assay, toxicity, and efficacy). This product, which showed a potentially better therapeutic effect in comparison with the marketed Phenergan®, has been further developed and is undergoing clinical trials in our laboratory. Our ideas and research could be applied in other pharmaceutical products to enhance the development of inexpensive medicines for developing countries.

ACKNOWLEDGEMENTS

DT Pham wishes to sincerely thank the Department of Pharmacology, Faculty of Pharmacy, Can Tho University of Medicine and Pharmacy, for supporting the animals and providing necessary help. The authors also appreciate the cooperation of senior pharmacy students.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):178-183
DOI: 10.4274/tjps.22931

ORIGINAL ARTICLE

Preparation and Characterization of Caffeine Loaded Liposome and Ethosome Formulations for Transungual Application

Transungual Uygulama için Kafein Yüklü Lipozom ve Etazom Formülasyonlarının Hazırlanması ve Karakterizasyonu

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ABSTRACT

Objectives: Nail plates have a structure that prevents transungual delivery of active agents. This situation makes it difficult to treat nail diseases.

Materials and Methods: In this study, CF-loaded liposome and ethosome formulations were prepared for ungual application. Formulations were characterized by size, microscopic observation, pH, and entrapment efficiency measurements. The effects of formulations and experimental conditions on nails were tested with characterization of nails before and after *ex vivo* permeation experiments.

Results: Microscopic observation confirmed the presence of spherical-structured vesicles. The particle sizes of vesicles were found as 545.3±0.121 nm, 610.2±0.943 nm, 349.5±0.145 nm and 337.9±0.088 nm for liposomes (FI-FII) and ethosomes (FIII- FIV), respectively. The polydispersity index of particles was found under 0.5, and the pH of formulations was around 7. The encapsulation efficiency was found low due to the hydrophilic character of CF. Nail characterization studies showed that the experimental conditions had an effect on the nail plate.

Conclusion: The cumulative amount of drug after *ex vivo* permeation studies was found higher for ethosomes than for liposomes. The results confirm that liposomal systems could be promising systems for ungual drug delivery.

Key words: Transungual delivery, nail, liposome, ethosome, caffeine

ÖZ

Amaç: Tırnak plağı, etken maddelerin tırnaktan geçişini engelleyen bir yapıya sahiptir. Bu durum tırnak hastalıklarının tedavisini zorlaştırmaktadır.

Gereç ve Yöntemler: Bu çalışmada, ungual uygulama için CF yüklü lipozom ve etazom formülasyonları hazırlanmıştır. Formülasyonlar, yükleme kapasitesi, partikül boyutları, mikroskopik görüntülemesi, pH değerleri ile karakterize edilmiştir. Formülasyonların ve deneysel koşulların tırnaklar üzerindeki etkisi *ex vivo* geçiş çalışmaları öncesinde ve sonrasında yapılan tırnak karakterizasyonları ile test edilmiştir.

Bulgular: Mikroskobik inceleme veziküllerin dairesel yapısını konfirme etmiştir. Partikül boyutları lipozomlar (FI-FII) ve etazomlar (FIII-FIV) için sırasıyla, 545.3±0.121 nm, 610.2±0.943 nm, 349.5±0.145 nm ve 337.9±0.088 nm olarak bulunmuştur. Partiküllerin polidispersite indeksi 0.5'in altında bulunmuştur ve formülasyonların pH'si 7 civarındadır. CF'nin hidrofilik karakterine bağlı olarak yükleme kapasitesi düşük bulunmuştur. Tırnak karakterizasyon çalışmaları deneysel koşulların tırnak plağı üzerinde etkisi olduğunu göstermiştir.

Sonuç: Sonuç olarak, *ex vivo* geçiş çalışmaları sonucunda birikimli ilaç miktarı etazomlarda lipozomlardan daha fazla bulunmuştur. Sonuçlar, lipozomal sistemlerin ungual ilaç taşıyıcı sistemler olarak umut verici sistemler olduğunu doğrulamıştır.

Anahtar kelimeler: Transungual geçiş, tırnak, lipozom, etazom, kafein

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Received: 21.06.2017, **Accepted:** 21.09.2017

INTRODUCTION

The human nail produces a strong, relatively inflexible nail plate, which is the most visible part of the nail apparatus.[1] The nail plate is derived from the epidermis but it is completely different in structure. In nail plates, keratin molecules are highly linked to disulfide bonds, which increase the thickness of the plates 100 times more than the epidermis.[2,3] Nail plates have 80-90 layers of dead cells and consist of hard and soft-type keratin, water, and a small amount of lipid.[4-6] Human nail plates behave like a hydrophilic gel membrane.[1,7,8] The structure of the nail plate prevents effective ungual treatment of nail disorders because active agents cannot permeate through nail plates.[9-13] In order to deliver a sufficient amount of active agent into and through the nail plate, the permeability of the nail plate to the drug needs to be enhanced.

Nail disorders can be treated with topical or oral therapies. Oral therapy, especially for fungal infections, has toxicity due to the long duration of treatment.[1,14] As mentioned above, topical therapy is limited with low drug permeation through the nail plate.[10,15] Therefore, patients are advised to apply formulations on the nail plate and surrounding tissue. Active drug can be eventually delivered through an alternative pathway, i.e., the surrounding skin above the nail plate, and the effectiveness is increased. In this approach, liposomes and ethosomes could be effective systems for transungual application of active agents. Liposomes are vesicular systems composed of phospholipids already present in the skin structure. Liposomes could enhance penetration of many drugs through the upper layer of the stratum corneum. Among their several possible pharmaceutic applications, they have been widely applied in topical drug delivery.[16-19] Liposomes cannot be used as transdermal delivery systems due to their low penetration through the deeper skin layers. Therefore, vesicular systems such as ethosomes, transfersomes or niosomes are delivered from liposomes. Ethosomes are vesicular systems that contain high concentrations of ethanol.[20] The presence of ethanol in systems of lipid vesicles influences penetration into the stratum corneum and the permeation of drugs.[21] The ethosomal system is composed of phospholipids, ethanol, and water. Liposomes and ethosomes have a lipophilic character and could use lipophilic pathways in the nail plate; they could lead to more effective treatments of nail disorders. In the present study, caffeine (CF) was chosen as a model drug owing to its water soluble character, an relatively low molecular weight (194.2 g/mol). CF-loaded liposome and ethosome formulations were prepared and formulations were characterized via encapsulation efficiency, particle size, and morphology. CF permeability through the human nail plate was examined. Thus, by comparison of the obtained permeability coefficients, the suitability of the formulations was evaluated. The effects of formulations and permeation conditions on human nail characteristics were studied and the most promising formulation is suggested.

MATERIALS AND METHODS

CF was purchased from Böhringer Ingelheim (Ingelheim am Rhein, Germany) and phosholipon 90 G and lipoid S 100, were gifts from lipoid AG (Steinhausen Switzerland). Cholesterol was purchased from Sigma Company (St. Louis, USA). All other chemicals and reagents used in this study were of analytical grade or higher and were obtained commercially. In this work, nail samples were used for *ex vivo* permeation studies. The human cadaver nail samples were collected from human corpses that had been used in anatomy courses of Institute of Anatomy and Cell Biology, Freiburg, Germany. Therefore, ethics committee approval was not needed and was not taken.

Preparation of liposomal formulations

Liposomes were prepared using the thin film hydration method.[18] Phospholipid, cholesterol and CF were dissolved in chloroform. Chloroform was then evaporated using a rotary evaporator (IKA RV10, Staufen, Germany) at 40 °C and 100 rpm. The flask was left under vacuum overnight to completely remove all traces of the solvent. The obtained thin film on the flask wall was hydrated with 10 mL phosphate-buffered saline (PBS, pH: 7.4). The liposomal suspension was homogenized using a polytron (Kinematica AG, Switzerland) at 13,000 rpm for 15 min. The compositions of liposomes (FI and FII) are shown in Table 1.

Preparation of ethosomal formulations

Ethosomal formulations were prepared according to the ethanol injection method (Table 1, FIII and FIV).[20] Phospholipid and CF were dissolved in ethanol and double-distilled water, respectively. The aqueous phase was added slowly to the lipid solution with constant stirring at 700 rpm. The system was kept at 30 °C. The resulting vesicle suspension was homogenized using a polytron (Kinematica AG, Switzerland) at 13,000 rpm for 15 min.

Characterization of formulations

The particle size and polydispersity index of the formulations were determined using a light scattering method with a Malvern Zetasizer 1000 HS (Malvern Instruments, England). The vesicular suspensions were diluted with PBS (pH: 7.4) for liposomes and 40% of the ethanol:water mixture for ethosomes before measuring particle size. The pH values of the formulations were determined using a pH meter (Jenway 3040 Ion Analyze) at 25±1 °C. All experiments were replicated

Table 1. Compositions of the formulations FI-FIV

Compositions	FI	FII	FIII	FIV
Caffeine	0.025 g	0.025 g	0.177 g	0.174 g
Lipoid S 100	0.222 g		0.15 g	0.3 g
Phospholipon 90 G		0.222 g		
Cholesterol	0.277 g	0.277 g		
Chloroform	12.5 mL	12.5 mL		
Ethanol			6 g	6 g
Water			8.673 g	8.526 g
PBS (pH: 7.4)	10 mL	10 mL		

PBS: Phosphate-buffered saline

at least three times. The formulations were visualized using transmission electron microscopy (TEM) (Philips, Amsterdam) to confirm vesicular structure. The samples were negatively stained with a 1.5% aqueous solution of phosphotungstic acid. The vesicular suspensions were placed onto a carbon-coated copper grid and the excess solution was removed with a filter paper. The grid was left in room conditions for air-drying and then the films were observed on the TEM. The entrapment efficiency (EE) of both methods regarding CF was measured using the dialysis bag method.[22-24] One milliliter of drug-loaded liposomes and ethosome dispersions were dropped into a cellulose acetate dialysis bag (Spectra/Por®, MW cut-off 12.000; Spectrum, Canada) immersed in 150 mL of water. The system was stirred at 400 rpm. After taking samples from the receiver solutions, fresh samples were added to obtain sink conditions. CF was spectrometrically assayed at 273 nm using an ultraviolet (UV)-spectrophotometer (UV-spectrophotometer DU720, Beckman Coulter, California, USA). The percent of encapsulation efficiency (EE %) was calculated according to the following equation.

$$EE\% = \frac{[\text{Total drug})-(\text{diffused drug}]}{[\text{total drug}]} .100 \quad \text{(Eq. 1)}$$

Preparations of nails

Human cadaver nail samples were collected from human corpses that had been used in anatomy courses of Institute of Anatomy and Cell Biology, Freiburg, Germany. The whole nail plate collection technique was previously reported by Tuncay Tanrıverdi, 2013.[25] Only "healthy" nail plates were taken for permeation studies. Nail samples were kept at -20 °C.

Characterization of nail samples

The nail plates were left at room temperature overnight to equilibrate. Before starting the permeation experiments, the nail plates were weighed and the thickness of plates was measured with using digital micrometer (Digit cal SI, TESA S.A, Renens, Switzerland). Transonychial water loss (TOWL) was measured (Tewameter TM210, Courage&Khazaka Electronic GmbH, Germany) and Visioscan images (Visioscan VC98, Courage&Khazaka Electronic GmbH, Germany) of the nail plates were taken. The nail plates were then immersed in PBS (pH: 7.4) for hydration. The characterization study was performed again after hydration in PBS and after the permeation studies. The nail plates were left to dry at room conditions after the permeation studies and characterization was performed one again.

Nail permeation studies

A nail adaptor was used to fix the nail plates on Franz diffusion cells with an area of 0.785 cm^2. The acceptor chambers were filled with 5 mL of PBS (pH: 7.4), and 400 µL of liposome and ethosome suspensions were added to the donor chamber. The receptor phase was constantly stirred using a magnetic stirrer at 400 rpm. The system was kept at 32 °C with a water bath and water jacket of the diffusion cells. Each day, a sample of 400 µL was taken from each cell over a period ten days and replaced with the same volume of PBS (pH: 7.4) warmed to 32 °C. The amount of drug in the sample was measured using a UV-spectrophotometer at 273 nm. The permeability coefficient, p (cm/s) was calculated from the CF flux in the steady state and the initially measured CF concentration of the applied formulation. The enhancement factor represents the permeability coefficient of a formulation divided by the permeability coefficient of ethanol: water and water solutions of CF such as a control. Thus, the possible enhancing effect of ethanol was eliminated. Additionally, the possible interference of ethanol on UV measurement was investigated and it was concluded that an enhancement factor did not notably change the results.

RESULTS AND DISCUSSION

Characterization of formulations

The CF-loaded liposomes and ethosomes (FI- FIV) were scanned using TEM and spherical structures were confirmed (Figure 1).

The particle size and the polydispersity of size distribution of the liposome and ethosome formulations measured by light scater are shown in Table 2. In accordance with other authors, the conventional liposomes showed a larger particle size and a higher polydispersity than the ethosomes.[26] Statistical analyses [One-way ANOVA, post-hoc Tukey honest significant difference (HSD) test] showed that the particle size of all formulations were significantly different ($p<0.01$). As reported by López-Pinto et al.[24], ethanol confers a surface negative net charge to vesicles, which causes a decrease in the size of vesicles. The ability of the vesicles to entrap CF was investigated. EE was determined to be rather low according to the hydrophilic character of CF. Significant differences were observed between the four formulations (One-way ANOVA, post-hoc Tukey HSD test, $p<0.01$).

Nail characterization

The nails were characterized via, weight, thickness, Visioscan images, and TOWL. The nails were characterized before the nail

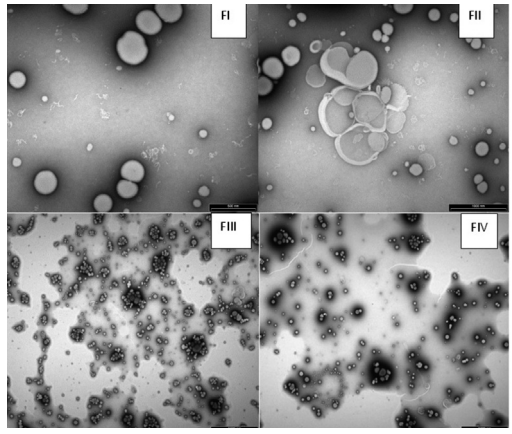

Figure 1. Transmission electron microscopy images of caffeine-loaded liposomes and ethosomes (FI, FII, FIII and FIV)

permeation experiment. Then nails were immersed in PBS and characterized after being taking out from PBS. The parameters were tested after the permeation study, and finally, the nails were kept at room temperature to dry for 24 h. After drying, the nail samples were characterized again. The weight of the nail plates increased after immersion in PBS for 1 h. The percentage of weight increase was found as 23.89%. The plates behave like a hydrophilic gel system. Therefore, plates could swell after immersion in solvent. However, 24 h after the experiment, the weight of nails decreased due to drying of the samples. It was concluded that the experimental conditions and room temperature could affect the weight of the nails. The thickness of the nails was also measured three times per nail. The nail thickness varies according to the person and the measured region as they are biological tissue. The nail samples were kept in PBS before the experiment. The sample thicknesses were re-measured after removal from PBS. The thickness of six nails increased, whereas the thickness of the other six nails decreased. The TOWL of the nails was measured before, after 1 h PBS immersion, after the experiment, and one day after the experiment. The results of TOWL are summarized in Table 3. The data showed that TOWL of the nails increased after 1 h immersion in PBS and after the experiment, as expected. On the day after the permeation experiment, the TOWL values of all nails decreased as was seen in the weight of nails. However, no correlation between TOWL and weight was found. This probably due to the decreasing water content during the 24 h, and then they became dry. The differences in TOWL can be partially assigned to the different thicknesses of the nail plates, as shown in Figure 2, where a relatively good correlation between thickness and TOWL was found before the permeation experiment.

The nail permeation experiments were continued for 10 days. During the experiments, the nails were treated with formulations and receptor phase at 32 ˚C. A Visioscan VC 98 was used to evaluate nail surfaces before and after experiment.[27] Figure 3 shows the dry surface of a nail plate before and 24 h after the experiment, which was treated with different formulations. The results showed that the application of liposomes and ethosomes induced a change of the superficial nail structure. Ethosomes had a greater effect on nail plates due to their high ethanol concentration.

Nail permeation studies

Table 4 shows the permeability coefficients and enhancement factors of the applied formulations. CF solutions in ethanol: water for ethosomes and in water for liposomes were used as control formulations. A significant improvement in permeability was found for both ethosomal formulations (One-way ANOVA, post-hoc Tukey HSD test, $p<0.01$). As expected, the molecular size of the vesicles had an inverse relationship with permeation through the nail plate (Figure 4). The ethanol/water solvents present in the system are suggested to hydrate the nail plate and

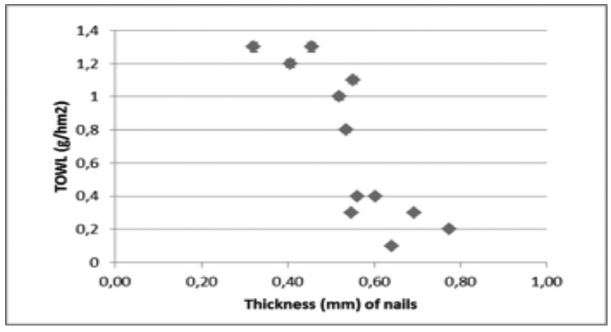

Figure 2. The influence of nail plate thickness on transonychial water loss

TOWL: Transonychial water loss

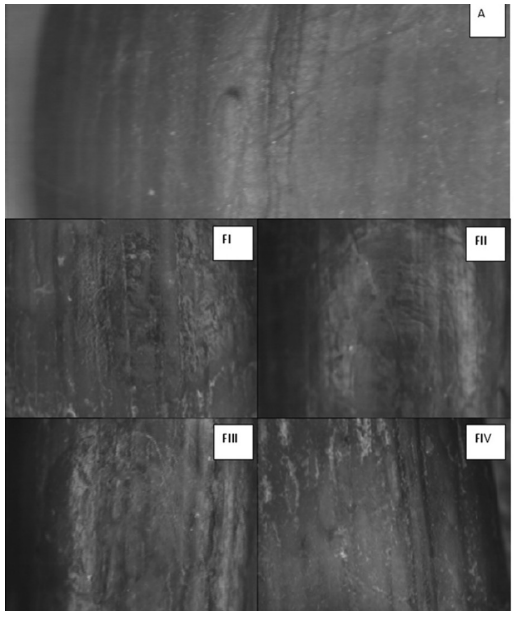

Figure 3. Visioscan images of nail before and after permeation experiment treated with FI-FIV formulations. A) The image of nail plate before the experiment

Table 2. Characterization of formulations

	FI	FII	FIII	FIV
Z-size (nm)	545.3±0.121	610.2±0.943	349.5±0.145	337.9±0.088
PI*	0.375±0.031	0.253±0.034	0.195±0.03	0.249±0.074
EE (%)*	7.17±0.14	16.95±0.21	9.92±0.12	18.40±0.63
pH	7.33±0.03	7.33±0.02	7.73±0.01	7.79±0.01

*PI, represents the polydispersity index used as indication of size distribution of vesicles, EE: Encapsulation efficiency, PI: Polydispersity index

Table 3. Transonychial water loss values of 12 nails

TOWL (g/hm²)*									
Liposome (FI-FII)					Ethosome (FIII-FIV)				
Nails	B.E*	A.PBS*	A.E.*	D.A.E.*	Nails	B.E*	A.PBS*	A.E.*	D.A.E.*
1	0.8±0.01	6.6±0.04	5.5±0.02	0.9±0.01	7	0.3±0.01	7.1±0.03	12.9±0.04	0.2±0.01
2	1.3±0.02	6.1±0.02	11.8±0.01	0.3±0.01	8	0.4±0.03	12.6±0.02	12.3±0.05	0.6±0.03
3	1.2±0.02	8.9±0.03	5.8±0.04	0.6±0.02	9	0.2±0.02	5±0.02	13.9±0.02	0.3±0.02
4	1.3±0.01	6.6±0.01	7.6±0.05	0.5±0.02	10	0.4±0.05	8.8±0.03	16±0.01	0.2±0.01
5	1±0.02	8.5±0.01	9.5±0.02	0.8±0.01	11	0.3±0.03	4.5±0.05	14.4±0.05	0.1±0.01
6	1.1±0.01	7±0.03	15.9±0.06	0.5±0.02	12	0.1±0.01	10.5±0.06	18±0.03	0.1±0.03

*B.E.: Before experiment, A.PBS: After immersing in phosphate-buffered saline for 1 h, A.E.: After experiment, D.A.E.: Day after experiment, TOWL: Transonychial water loss

Table 4. Permeability coefficient and enhancement factor of formulations and references

Formulations	Permeability coefficient (x10⁻⁸ cm/s)	Enhancement factor
FI	3.4±2.007	2.18
FII	5.5±1.31	3.53
FIII	17.953±6.148	10.20
FIV	14.9±5.142	8.47
Control CF-E/W	1.56±0.85	1.00
Control CF-W	1.76±0.79	1.00

CF: Caffeine

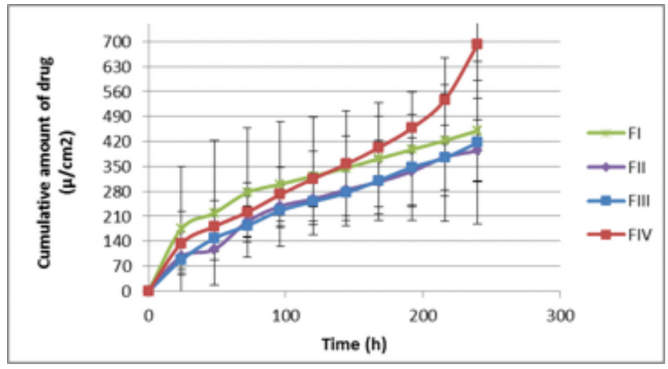

Figure 4. Cumulative amount of drug in receptor phase during the permeation experiment

thus increase permeation of CF. Kobayashi et al.[28] suggested that permeability through healthy and fungal nail plates is not significantly different. Thus, fungal nail permeability can be estimated from healthy nail permeability data. They also noted that the flux of drug through very heavily infected fungal nail plates might be higher than through healthy nail plates.

CONCLUSION

Nail disorders cannot be successfully treated with topical therapy due to the hard structure of nail plates. The nail plate is a barrier for the penetration of active substances. The aim of the study was to investigate the penetration enhancing effect of liposome and ethosome formulations for transungual delivery. CF was chosen as a hydrophilic drug. In this study, CF loaded liposomes and ethosomes were prepared for the first time for ungual application. Also, the effects of formulations and *ex vivo* permeation study conditions on nail plates were recorded via several parameters. CF was loaded with ethosomes and liposomes with low loading capacity because of the hydrophilic character of active agent. The particle size of ethosomes was found lower than liposomes. According to *ex vivo* studies, it was concluded that ethosomes had a greater penetration enhancing effect than liposomes. Finally, nail characterization studies showed that the experimental conditions had an effect on nail properties. As a conclusion, hydrophilic-drug-loaded liposome and ethosome formulations were prepared and characterized. The results suggest that liposomes and ethosomes could enhance penetration of hydrophilic substances through nail plates and both systems can be used for topical treatment of nail disorders.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):184-189
DOI: 10.4274/tjps.52824

ORIGINAL ARTICLE

Comparative Clinical Study of the Effectiveness of MEKRITEN in Patients with Chronic Suppurative Otitis

Kronik Süpüratif Otitis Medialı Hastalarda MEKRITEN Etkinliğinin Karşılaştırmalı Klinik Çalışması

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ABSTRACT

Objectives: Chronic suppurative otitis media (CSOM) is a serious disease with the presence of bacterial infection in the middle ear. Worldwide, 1% to 46% of the population living in developed and developing countries have CSOM, about 65-330 million people, 60% of whom have significant hearing loss. The study of the wound healing efficacy and tolerability of the drug MEKRITEN (garlic extract liquid) was performed to identify the possibility of issuing recommendations for the drug for clinical use in the Republic of Uzbekistan.

Materials and Methods: The main group of patients, who received the study drug, consisted of 30 patients; the group of patients who received the comparison drug comprised 20 patients. Patients of the main group (30 persons) were given 'garlic extract liquid,' which was developed by the Tashkent Pharmaceutical Institute, given as 2 drops twice per day for 10 days in the external auditory canal. Patients in the comparison group (20 people) took other drugs (0.25% solution of levomycetin) in a similar way. Patients were examined and observed carefully to determine the severity of symptoms such as pain, secretion (in points), and clinical analyses were performed including general blood analysis, and urine. In addition, the researchers performed biochemical (ALT, AST, bilirubin), instrumental (ultrasound of the liver), and special (biopsy of fibrous tissue of echinococcal capsules) analyses.

Results: The average rating of the tolerability and effectiveness in the points for MEKRITEN made portability of 4.97 points, and the effectiveness was 4.77 points, and for 0.25% levomycetin solution, they were 4.8 and 3.35 points, respectively. While receiving MEKRITEN, no adverse effects and allergic reactions were observed. No criteria for treatment cessation were observed (adverse effects, ineffective treatment, poor adherence to protocol, and refusal to continue) thus the study was not stopped in any patients.

Conclusion: Local therapy with the drug MEKRITEN in patients with CSOM is more effective than local application of 0.25% levomycetin solution. Local therapy with MEKRITEN in the treatment of CSOM leads to faster termination of otorrhoea and is accompanied by fewer adverse drug reactions.

Key words: MEKRITEN, levomycetin, chronic suppurative otitis media, garlic extract liquid

ÖZ

Amaç: Kronik süpüratif otitis media (KSOM), orta kulakta bakteri enfeksiyonu bulunan ciddi bir hastalıktır. Dünya çapında, gelişmiş ve gelişmekte olan ülkelerde yaşayan nüfusun %1-46'sı, yaklaşık 65-330 milyon insan, KSOM'dan muzdarip olup, bunların %60'ında ise önemli işitme kaybı vardır. Özbekistan Cumhuriyeti'nde klinik kullanım için ilaç önerileri verme olasılığını belirlemek için MEKRITEN (sarımsak özü sıvısı) ilacın yara iyileşme etkinliği ve tolere edilebilirliği araştırılmıştır.

Gereç ve Yöntemler: Çalışma ilacını alan ana grup 30 hastadan, karşılaştırma ilacı alan hasta grubu 20 hastadan oluşmaktadır. Ana grubun (30 kişilik) hastalarına, Taşkent İlaç Enstitüsü tarafından geliştirilen sarımsak özü sıvısı 10 gün süreyle dış kulak yolu kanalına günde 2 damla olarak verildi. Karşılaştırma grubundaki hastalar (20 kişi) benzer şekilde diğer ilaçları (%0.25 levomisetin çözeltisi) aldı. Hastalar, ağrı, sekresyon (puan olarak) gibi şikayetlerin ciddiyetini belirlemek için dikkatli bir şekilde incelendi ve genel kan ve idrar analizi dahil olmak üzere klinik analizler yapıldı. Ayrıca gözlemciler biyokimyasal (ALT, AST, bilirubin), enstrümantal (karaciğer ultrasonu) ve ekineokoksik kapsüllerin fibröz dokusunun biyopsi analizlerini inceledi.

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Received: 19.01.2017, Accepted: 18.05.2017

Bulgular: MEKRITEN puanlarındaki tolere edilebilirlik ve etkililiğin ortalama derecelendirmesi, 4.97 puanlık taşınabilirlik ve 4.77 puanlık etkililiği %0.25'lik levomisetin çözeltisi için sırasıyla 4.8 ve 3.35 olarak bulunmuştur. MEKRITEN alınırken yan etki ve alerjik reaksiyon gözlenmedi Tedavi kesilmesi için herhangi bir kriter gözlenmedi (yan etkiler, etkisiz tedavi, protokole uyumsuzluk ve devam etmeyi reddetme) bu nedenle hiçbir hastada çalışma durdurulmadı.

Sonuç: KSOM hastalarında MEKRITEN ile yapılan lokal terapi, %0.25'lik levomisetin solüsyonunun lokal uygulanmasından daha etkilidir. KSOM tedavisinde MEKRITEN ile lokal terapi, otorri'nin daha hızlı sonlanmasına yol açar.

Anahtar kelimeler: MEKRITEN, levomisetin, kronik süpüratif otitis media, sarımsak özü sıvısı

INTRODUCTION

Chronic suppurative otitis media (CSOM) is a serious disease with the presence of bacterial infection and perforated tympanic membrane with persistent drainage from the middle ear. It is a major cause of acquired hearing impairment in children, especially in developing countries. Most approaches to treatment have been unsatisfactory or are very expensive and difficult; for example, parenteral aminoglycosides require long hospitalization and are potentially ototoxic.[1] The pathologic process in chronic purulent otitis media leads to destruction of bone structures of the middle ear and causes hearing loss. Despite the use of antibacterial therapy, CSOM remains the main cause of hearing loss.[2]

CSOM is one of the most urgent problems of otorhinolaryngology because it is a significant part of the whole pathology of ear, nose and throat (ENT) organs, and ranks second in the structure of otorhinolaryngology morbidity. Prevalence surveys, which differ widely in disease definition, sampling methods, and methodologic quality, show that the global burden of illness from CSOM involves 65-330 million individuals with draining ears, 1% to 46% of the population of developed and developing countries have CSOM, 60% of whom suffer important hearing impairment. Annually, 31 million new cases of CSOM are registered in the world, 22.6% of which are diagnosed in children aged under 5 years. In 30.82 cases per 10,000, the disease is accompanied by hearing loss. Worldwide, 28,000 people die from complications of CSOM every year, mostly from intracranial complications.[2]

Among all chronic diseases of ENT organs, CSOM is the most frequent pathology (up to 48.8%). Among patients with ENT pathology who are assisted in the ENT departments, 5.7-7% have CSOM. CSOM with frequent exacerbations is the cause of ontogeny complications that currently appear in 3.2% patients, intracranial complications are observed in 1.97% (e.g., meningitis, brain abscess) and extracranial complications in 1.35% (e.g., subperiosteal abscess, labyrinthitis). Mortality from CSOM is 16-30%. One of the reasons of the development of destruction in the middle ear is cholesteatoma, which is found in 24-63% of patients with CSOM at any location of the perforation of the eardrum. Both acute and CSOM are still one of the most common diseases of ENT organs, ranging from 5.1 to 58% of cases, and are the cause of high levels of hearing loss in 8.6 to 37% of cases.[3,4]

The issues of early diagnosis, choice of treatment, and characteristics of patients with CSOM are still relevant. However, the solution for these problems is closely connected with the study of various aspects of the etiopathogenesis of the disease, including general and local immunologic reactivity, the state of the antioxidant system of the organism, i.e. the background, where there is probably a pathologic process.

In connection with the above, the efficacy of oil extract of garlic, which was developed by the Tashkent Pharmaceutical Institute, was investigated. In preclinical studies, the drug showed high efficacy in experimental models.

Purpose: To study the wound healing efficacy and tolerability of the drug 'garlic extract liquid' developed by the Tashkent Pharmaceutical Institute, Uzbekistan, and to identify the possibility of issuing recommendations for the drug for clinical use in the Republic of Uzbekistan (RUz).

MATERIALS AND METHODS

This study was open, full designed, and performed on two parallel groups. The main group of patients that received the new drug consisted of 30 patients. The second group of patients, which received the comparison drug, comprised 20 patients. The groups were matched by sex, age, and diagnosis. Patients of both sexes receiving outpatient treatment, aged over 18 years, and who gave written informed consent for participation in the research, and those who underwent surgery in the middle ear (myringoplasty) were included in the study.[2]

The criteria for exclusion were patients aged less than 18 years, pregnancy, lactation, the presence of hypersensitivity to the drug component, participation in other clinical trials within the last 30 days, no written informed consent for participation in clinical research, and contraindications to the use of the drug.

Patient details

The first group included 30 patients with a mean age of 34±12.33 years; 13 patients were women and 17 were men. Eight patients were diagnosed as having right-sided CSOM, 11 patients were hospitalized with left CSOM, and the remaining 11 patients had bilateral CSOM.

Second group consisted of 20 patients and their average age was 35±13.42 years. Twelve patients were female and 8 were male. Ten patients were diagnosed as having right-sided CSOM, 6 had left CSOM, and 4 patients were treated for bilateral CSOM.

The scheme of drugs appointment

Patients of the main group (30 persons) were given garlic extract liquid. The medication was given as 2 drops twice per day for 10 days in the external auditory canal. Patients in the comparison group (20 people) took other drugs (0.25% levomycetin solution) in a similar way. Simultaneous complementary therapies were not performed. Other drugs with a similar action were excluded.

Medications and other drugs that are compatible with the drug, as well as the necessary physical therapy, were used for the necessary treatment of the underlying disease.

The overall schedule of the research

- After the initial examination of patients who met the inclusion criteria, in order to obtain their written informed consent to participate in the study, the patients were provided with information about the experimental drug 'garlic extract liquid,' information about doses, schemes, routes of administration, and period of treatment.

- When the patients' written consent for participation in the research was obtained, they were administered the study drug or the comparison drug.

- The starting point of the patient's participation in the study: the first day of receiving study drug or the comparison drug.

- Treatment was described in all patients included in the study.

- Any treatment related to concomitant diseases was registered in the medical history and individual registration form.

The patient was provided with the informed consent form for participation in a clinical trial. The patient had enough time for decision-making. The patient signed the informed consent. Procedures for verification of patient compliance of doctor orders are governed by the internal regulations of hospital, where the study was conducted. If there was a break in the procedure of taking the drug, the patient was excluded from the study.[3]

Responsible person will not allow the use of the test drug and comparison drug for any other purpose except that specified in the protocol of a clinical trial.

After the completion of the study, a report on the use of the test drug and comparison drug was made using Form 2 of Appendix No: 2 to the order of Ministry of Health (MH) of the RUz No: 334, dated: 25.07.2001.

Examination

Patients were examined and observed carefully to determine the severity of symptoms such as pain, secretion (in points); and clinical analyses were checked, including general blood analysis and urine. In addition, the researchers performed biochemical (alanine aminotransferase, aspartate transaminase, bilirubin), instrumental (ultrasound of the liver) and special (biopsy of fibrous tissue of echinococcal capsules) analyses.

Criteria for evaluating the effectiveness of the study drug

The list of performance indicators:

- The complete disappearance of the inflammatory process and pain,

- Normalization of laboratory and instrumental studies.

Evaluation of the effectiveness of an investigational drug was conducted by the researcher on the basis of the above criteria in points according to the following scale (Table 1):

Methods and timing of assessing, recording, and statistical processing of performance indicators:

Registration of the performance indicators was conducted immediately after examination and/or receipt of laboratory data. The information expressed in a quantitative form was subjected to statistical processing, including the use of special software.

Student's t-test is assumed for the application of methods of variation statistics. If necessary, multivariate analysis was performed.[4]

Table 1. Criteria for an assessment of efficiency of the study drug

3 points	High efficiency	A marked disappearance of symptoms: no pain and secretions (points 0-2), Normalization of the indicators of laboratory and instrumental studies at the end of the test.
2 points	Reasonable efficiency	Moderate disappearance of symptoms: moderate pain reduction and secretions (points 3-4), a moderate improvement in the indicators of laboratory and instrumental studies at the end of the test.
1 point	Low efficiency	The disappearance of minor symptoms: a small decrease in pain and secretions (points 5-6), a slight improvement of indicators of laboratory and instrumental studies at the end of the test.
0 point	Lack of efficiency	No change or worsening of clinical and laboratory parameters at the end of treatment.

Criteria for assessing the tolerability of the study drug

Tolerability was assessed on the basis of subjective symptoms and sensations reported by the patient, and objective data obtained by the researcher in the treatment process. The dynamics of laboratory indicators and the incidence and nature of adverse reactions were taken into account.

Tolerability was assessed and scored by the researcher and patients (Table 2).

RESULTS

Efficacy: analysis of efficacy was performed on the results of the study on patients receiving the drug according to the scheme provided in this protocol.

The results are provided in Table 3.

Tolerability: When analyzing adverse effects, it is necessary to exclude adverse effects that might occur from taking other medications or treatment procedures prescribed to the patient along with the test drug. If there is uncertainty, this case can only be partly analyzed regarding intolerance.

The average rating of the tolerability and effectiveness for MEKRITEN found portability as 4.97 points, and the effectiveness as 4.77 points, compared with 0.25% levomycetin solution at 4.8 and 3.35 points, respectively (Table 4).

Neither drug had any adverse effects on the blood according to a general analysis.

Table 2. Assessment of tolerability of patients to the study drug (in points)

4 points	During objective examination and/or laboratory research in the dynamics any pathologic changes or clinically significant deviations are not revealed and/or a patient does not note adverse reactions
3 points	During objective examination and/or laboratory research in the dynamics minor changes are revealed that are transient in nature and do not require change of treatment with investigational drugs and/or a patient notes symptoms of minor adverse reactions, which are not causing serious problems.
2 points	During objective examination and/or laboratory, research in the dynamics significant changes are revealed that do not require additional measures and/or a patient notes an adverse reaction, which has a negative impact on his condition, but does not require discontinuation of the drug.
1 point	During objective examination and/or laboratory research in the dynamics substantial changes are revealed, and/or the patient notes an adverse reaction which has negative effects on his condition and require discontinuation of the drug
0 point	During objective examination and/or laboratory research in the dynamics significant changes are revealed and/or the patient notes an adverse reaction requiring discontinuation of the drug and additional medical measures

Table 3. Efficacy of MEKRITEN

Indicator	MEKRITEN		Levomycetin	
	Before	After	Before	After
Pain	0.033	0	0	0
Secretions	2.27	0	2.35	0.9

Monitoring, audit and inspection

In the study, monitoring and auditing is made by the Customer; during the study and at the end of it inspection from the Pharmacological Committee of the MOH and GDQCM (General Directorate for Quality Control of Medicines) of the RUz is possible.

The form of release - in bottles.

Information about "MEKRITEN"

Preparation of Garlic Extract contains the oil extract of garlic as the active substances. Release form - in bottles. Storage conditions - In a dry, cool place. Shelf life - 2 years. Preparation for research and the preparation of the comparison is provided by the Contractor to the Customer free of charge. Test samples should not be used otherwise than for specified clinical studies. While receiving MEKRITEN, no side effects and allergic reactions were observed.

Report registration

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. Clinical and analytical data obtained during this study should be evaluated and presented spreadsheets, and then summarized and discussed in the report prepared in accordance with Annex 2 to the order MOH of RUz No: 334 dated 25 July 2001. This study was investigated and approved by Clinical Ethical Committee of MOH, RUz and registered N.16/2 with the date of 02.05.2014.

Record keeping

Clinical trial documents in accordance with the list of documents of clinical trials that must be stored in a clinical database (add-on 4. to the item 5.3 of the Annex No: 1 to the Order of MH of the Rep Uzb. No: 334 dated 25 July 2001) should be retained for at least 15 years.

Statistical analysis

All the parameters measured are expressed as means ± standard deviation. The difference between the mean values was analyzed using Student's t-test at the 5% significance level. $P<0.05$ was considered to be significant, and $p>0.05$ was non-significant.

Table 4. Changes of indicators of general blood analysis with MEKRITEN and 0.25% levomycetin

No	Indicators	MEKRITEN		Levomycetin	
		Before	After	Before	After
1	Hemoglobin	113.4 g/l	115.4 g/l	114.4 μ/l	114.4 g/l
2	Erythrocyte	$4.14x10^{12}$/l	$4.19x10^{12}$/l	$4x10^{12}$/l	$3.9x10^{12}$/l
3	Leukocyte	$8.93x10^{9}$/l	$5.73x10^{9}$/l	$8.9x10^{9}$/l	$7.7x10^{9}$/l
4	ESR	7.07 mm/h	5.13 mm/h	7 mm/h	6 mm/h

ESR: Erythrocyte sedimentation rate

DISCUSSION

Garlic has been known for some time to have anti-infective properties against a wide range of microorganisms.[1] The present study has further demonstrated the antimicrobial potency of 'garlic extract liquid' against local multidrug-resistant bacteria and Candida isolates from Uzbekistan. The results of the patients using the brand-new drug MEKRITEN and levomycetin were comparable, thus showing that the isolates exhibited susceptibility. This indicates that 'garlic extract liquid' has a broad spectrum of antimicrobial activity and a wide therapeutic window. The isolates tested in this study are responsible for many diseases in Uzbekistan, including bacterial meningitis, maxillary sinusitis, and otolaryngologic diseases by *S. pneumoniae*, *H. influenza* and *S. pyogenes*,[2] bronchopulmonary disorders and CSOM by *Pseudomonas aeruginosa*,[3] candidiasis and vaginitis by *Candida albicans*,[4] nosocomial infections and bacteremia due to multidrug-resistant staphylococcal infections and diarrheal diseases caused by *Escherichia coli*, *Shigella* spp. (*S. dysenteriae*, *S. flexneri*, *S. boydii* and *S. sonnei*) and *Salmonella typhimurium*.[2,5] The sensitivity of these isolates to 'garlic extract liquid' also suggests that the intrinsic biosubstances in this extract are naive to the various drug resistance factors of the isolates, which include beta-lactamase expression, increased pyrrolidonylarylamidase activity, aminoglycoside-modifying enzymes, and altered ribosomal binding.[5] Meanwhile, the antimicrobial potency of garlic has been attributed to its ability to inhibit toxin production and expression of enzymes for pathogenesis.[1,4,5] Several studies previously demonstrated the antibacterial potency of 'garlic extract liquid' against enteropathogens such as *Vibrio parahaemolyticus*, *E. coli*, *Klebsiella pneumonia*; *Proteus* spp. (*P. mirabilis*, *P. vulgaris*, *P. penneri*, and *P. hauseri*); and *Staphylococcus aureus*,[6] and anticandidal effects against *Candida* spp. (*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis* and *C. crusei*.[7] In spite of geographic variation, 'garlic extract liquid' for our isolates are consistent with those of Sivam,[8] but are relatively lower than values obtained in the literature.[9] This antimicrobial potency disparity of garlic has been attributed to the different concentrations of individually and synergistically active biosubstances in garlic preparations coupled with their interactions with sulfhydryl agents in culture media. This phenomenon has been used to explain the stronger antimicrobial effect of allicin than garlic oil disulfides.[10] Allicin and other diallyl sulfide compounds have been found at different concentrations in MEKRITEN determined by age and method of extract preparation. It can be said that the concentration at which 'garlic extract liquid' showed growth inhibition was also fungicidal to our isolates because it displayed comparable MEKRITEN and nystatin values. The susceptibility response observed of some of the *P. aeruginosa* isolates aligns with the finding[10] but at variance with the work of Kivanc and Kunduhoglu.[11] The significantly increased minimum inhibitory concentration at 48 hours postinoculation may be the consequence of colony-forming unit rebound and the bacteriostatic effect of garlic extract liquid on these strains. The reliability of the clinical interpretation of this observation may undoubtedly require further tolerability testing of 'garlic extract liquid' in humans as a prelude to understanding garlic-induced plasma resistance to infections. The observation that 'garlic extract liquid' elicited its antimicrobial potency in a dose- and time-dependent manner producing distinct time-kill profiles suggests variations in the growth inhibitory responses of the tested isolates to garlic. Similar responses have been observed and surveyed in antibiotic-resistant *Escherichia coli*, *Enterobacter cloacae*, and *Citrobacter freund*.[12] However, the uniqueness of time-kill profiles of Gram-positive and Gram-negative bacteria may be connected with their structurally different cell wall barriers. The lipid composition of the cell wall has been found to have an influence on the permeability of hydrophobic and volatile bioactive substances in garlic.[13] The eukaryotic nature and ergosterol availability in *Candida* cell walls may also be crucial to the observed time-kill kinetics of 'garlic extract liquid' against our isolates.[14]

CONCLUSIONS

Local therapy with the drug MEKRITEN (garlic extract liquid) in patients with CSOM is more effective than local application of 0.25% levomycetin solution. Local therapy with MEKRITEN in the treatment of CSOM leads to faster termination of otorrhoea and is accompanied by fewer adverse drug reactions.

According to the results of clinical trials, ototoxic properties of the drug MEKRITEN in patients were not identified. The drug is well tolerated. MEKRITEN is fully comparable to levomycetin. Thus, MEKRITEN is effective in the treatment of CSOM and is recommended for medical use in the RUz.

ACKNOWLEDGEMENTS

This study was supported by Tashkent Pharmaceutical Institute, Tashkent, RUz. We also thank junior scientific researcher Bekhzod Abdullaev, who is a student of Tashkent Medical Academy, for his help in initiating the study, the artwork performed in the manuscript and the English correction.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):190-199
DOI: 10.4274/tjps.79188

ORIGINAL ARTICLE

Traditional Techniques Applied in Olive Oil Production Results in Lower Quality Products in Northern Cyprus

Kuzey Kıbrıs'ta Zeytinyağı Üretiminde Kullanılan Geleneksel Yöntemler Daha Düşük Kaliteli Ürünler Oluşturmaktadır

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ABSTRACT

Objectives: Olive oil production and its consumption is one of the traditional characteristics of Northern Cyprus. To date, no research has been conducted to analyze the quality of traditionally produced olive oil. Therefore, within this study, we aimed to analyze the olive oil produced within the island concomitant to the determination and comparison of its quality indices.

Materials and Methods: The standard olive oil analysis techniques acknowledged by the IOOC and ISO were employed. Accordingly, the fatty acid content, peroxide level, total phenol content, the levels of carotenoids and chlorophyll, as well as status of oxidation were all tested concomitant to statistical analysis.

Results: In contrast to the regional belief and consideration, the results indicated that the olive oil produced locally is highly exposed to oxidation and therefore, it is of lower quality according to the ISO guidelines.

Conclusion: The traditional techniques employed for the production, distribution, and storage of olive oil within Northern Cyprus must be re-evaluated and controlled to satisfy the current standards required and employed globally.

Key words: Olive oil, analysis, oxidation, Northern Cyprus

ÖZ

Amaç: Zeytinyağı üretimi ve tüketimi Kuzey Kıbrıs'ın geleneksel özelliklerinden biridir. Şu ana kadar, bu geleneksel olarak üretilen zeytinyağının kalitesini analiz etmek için yapılan bir bilimsel araştırma çalışması bulunmamaktadır. Bu nedenle, bu çalışmada, adada üretilen zeytinyağını analiz ederek kalite endekslerinin belirlenmesini ve karşılaştırılmasını amaçladık.

Gereç ve Yöntemler: IOOC ve ISO tarafından onaylanan standart zeytinyağı analiz teknikleri kullanılmıştır. Buna göre, yağ asiti içeriği, peroksit seviyesi, toplam fenol içeriği, karotenoidler ve klorofil seviyeleri ve oksidasyon durumu istatistiksel analizlere paralel olarak analiz edilmiştir.

Bulgular: Zeytinyağının Kuzey Kıbrıs'taki üretimi, dağıtımı ve depolanması için kullanılan geleneksel teknikler, global düzeyde ortaya konulan standartları karşılamak üzere yeniden değerlendirilmeli ve kontrol edilmelidir.

Sonuç: Bölgesel inanç ve değerlendirmenin aksine, sonuçlar, yerel olarak üretilen zeytinyağının oksidasyona aşırı derecede maruz kaldığını ve dolayısıyla ISO talimatlarına göre daha düşük kalitede olduğunu ortaya koymuştur.

Anahtar kelimeler: Zeytinyağı, analiz, oksidasyon, Kuzey Kıbrıs

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Received: 21.04.2017, **Accepted:** 22.06.2017

INTRODUCTION

There is no doubt that olive oil consumption has been a significant component of the daily diet in the Mediterranean region.[1] Indeed, olive tree (*Olea europea* L.) agriculture, and the following olive oil production is typical and one of the oldest traditions in various countries in the region.[1,2] Starting from the second half of the last century, the adoption of the Mediterranean diet in non-Mediterranean areas has led to the production of olive oil in higher amounts to respond to the worldwide demand which, in turn, has forced the establishment of industrialization for olive oil production to guarantee quality.[3,4] This has further been regulated and warranted by both the producer countries and the International Organization for Standardization.[5-7]

As a part of Cyprus, Northern Cyprus, also referred to as the Turkish Republic of Northern Cyprus, in the middle of the Mediterranean Sea, is one of those countries continuing to harvest thousands of tons of olive fruit and olive oil annually. Even archaeologic ruins from the Neolithic period (8200 BC) in the region indicate the processing of olive fruits since that time.[8] Records currently estimate the presence of approximately 1 million olive trees (*Olea europea* L.), many of which are cultivated to obtain olive fruits (i.e., a typical breakfast diet), and olive oil.[9] Furthermore, current data also indicate that olive oil consumption per person is around 25-50 mL/day in the region.[10] Indeed, almost all of the local restaurants in the region serve olive oil as one of the appetizers, even without charge.

Besides the existence of a few producers that use modern industrial subsidiaries, the majority employs traditional methods for olive oil production in Northern Cyprus. In public, these techniques are classified as either hot or cold procedures. As implied, cold and hot refers to the temperature in the extraction phase (i.e., a lower than 28˚C aqueous phase for the cold procedure and an above 28˚C aqueous phase for the hot procedure). It is considered that the hot procedure accelerates the extraction phase, therefore aids in the yield and stability with respect to the time spent. On the other hand, it is a disadvantage for the transfer of various beneficial chemicals (e.g., stabilizers, and antioxidants) to the aqueous phase depending on the change of solubility at varying temperatures.[11-13] Moreover, the majority of the public rely on olive oil products produced through traditional techniques rather than industrial products because they consider it more natural. The industrial local products are generally produced for export to other countries concomitant to analysis certificates guaranteeing the quality, whereas almost all of the local traditionally produced products reach consumers, even without an apparent label. The main difference with the current traditional techniques is that they do not obey the manufacturing rules strictly regulated in the industrialized techniques. The exposure to air and light during the production phase, the non-employment of protective equipment or systems to prevent oxidation and the related degeneration of olive oil, as well as the inadequate containers used (e.g., non-opaque glass material without a correct label) make the traditionally produced olive oils susceptible. Some producers still use stone-mills for malaxation.

To date, no scientific research has been conducted to screen the quality indices of traditionally produced olive oil in Northern Cyprus. From this point of view, this research aimed to investigate, for the first time, the basic quality parameters of the olive oil produced employing the traditional techniques within Northern Cyprus. Therefore, the quality indices [i.e., free fatty acid (FFA) percentage, peroxide value (PV), ultraviolet (UV)-specific extinctions at 232 and 272 nm, total phenol, chlorophyll, and carotenoid contents, and fatty acid alkyl ester (FAAEs) compositions] of the samples collected.

MATERIALS and METHODS

Chemicals and reagents

Hexane (99.0%), cyclohexane (99.5%), ethanol (99.9%), methanol (99.9%), diethyl ether (99.0%), sodium carbonate, sodium thiosulfate, potassium hydroxide (85.0%), potassium iodide (99.0%), sodium hydroxide (99.0%), acetic acid (99.0%), hydrochloric acid (37%), gallic acid were obtained from Merck (Germany). Folin-coicalteus phenol was purchased from Sigma-Aldrich (Darmstadt, Germany).

Samples

Although there are quite a number of producers, particularly within the western part of the region, the majority produce limited amounts for their own use. Therefore, we collected olive oil samples from the thirteen olive oil producers who sell their traditionally produced olive oils besides having it for their own use. From this point of view, 26 samples in capped non-opaque glass materials (i.e., the classic way of packing and marketing of producers) from 13 different producers were purchased directly from the producers (i.e., 2 samples from the same production of the same producer). Each sample from 13 producers was subcategorized in such a way that the first group was analyzed in their 3rd month of production, and the second group would be analyzed in their 6 month of production. The samples to be analyzed in these periods were kept in light-free shelves at room temperature until analysis. In order to make a comparison with a reference product from industrial production, a commercial extra virgin olive oil from the 3rd month of its production (the data on the label was used directly) was also purchased from a supermarket and employed in the same analysis. Each sample was analyzed three times and the results are expressed as mean ± standard deviation. Table 1 shows the codes, the place obtained, and the production method (i.e., cold or hot) of the samples analyzed.

Determination of FFA content

FFA, expressed as the percentage content of the FFA in olive oil, was determined through titration using potassium hydroxide according to the proposed procedure by ISO660.[14] Accordingly, a 500-mg olive oil sample was dissolved in 15 mL of ethanol and diethyl ether (solvent mixture), which was previously neutralized using potassium hydroxide. This solution was then titrated with 0.1N potassium hydroxide. Acidity, expressed as a percentage of fat type, was calculated according to the given formula:

Acidity= (V×N×F×M)/(10×m)

wherein:

V= The volume of 0.1N KOH consumed,

F= Factor of 0.1N KOH,

N= Normality of KOH (i.e., 0.1),

M= Molar mass of oil in gram per mole (i.e., 256 g/mol) and,

m= the mass in gram of the test portion.

Assessment of PV

PV, as stated in milliequivalent of O_2.kg^{-1} (meq O_2/kg oil), was determined according to the method described by ISO3960.[15] Briefly, a 5-g olive oil sample was dissolved in glacial acetic acid-hexane (6:4) solution. Then, 0.5 mL of saturated potassium iodide was added and swirled for exactly one min. Immediately after, 100 mL distilled water was introduced to the flask and shaken vigorously. Finally, the mixture was titrated with 0.01 N sodium thiosulfate. PV (meqO_2/kg oil) was calculated based on the formula described below:

PV= [(V-V_0)×N×F×1000]/m

wherein;

V: The volume of sodium thiosulfate consumed for the sample,

V_0: The volume of sodium thiosulfate consumed for the titration of the blank (without olive oil sample),

N: The normality of sodium thiosulfate (i.e., 0.01N), and,

m: Mass (weight) of sample in gram.

Determination of oxidation status of olive oils (K232 and K270)

The experiment to determine the oxidation status of olive oils was performed by measuring their absorption at specific wavelengths (i.e., 232 and 270 nm).[16] In brief, 0.25 g olive oil sample was dissolved in cyclohexane in a 25 mL graduated flask to prepare 1% w/v. Then, the specific extinctions at 232 and 270 nm were examined.

Carotenoids and chlorophyll content assays

Carotenoid and chlorophyll (mg/kg of oil) contents were determined employing a UV-based procedure.[17] As described above, a 0.25-g olive oil sample was dissolved in cyclohexane (i.e., 1% w/v) and the specific extinctions were determined at 470 and 670 nm, respectively, for the carotenoid and chlorophyll contents.

Detection of total phenol content (TPC)

The Folin-Ciocalteu method, an assay in which the results are expressed in terms of gallic acid as mg of gallic acid/kg olive oil depending on the spectrophotometric measurements conducted at 765 nm, was employed for the determination of the TPC.[18] Accordingly, 10 g of olive oil was dissolved in 50 mL of hexane and extracted three times with 80% aqueous methanol. The extract was then added to distilled water to a final volume of 100 mL aqueous methanol and kept overnight. Five milliliters of Folin-Ciocalteu phenol reagent was added to 1 mL of aliquot extract, then shaken well and left to stand for 5 min. One milliliter of saturated sodium carbonate was added and swirled. After 1 hour at room temperature, absorption was read at 765 nm. A one milliliter aliquot of 0.05, 0.2, 0.4, 0.5, and 0.6 mmol/L aqueous gallic acid solutions were mixed with 5 mL Folin-Ciocalteu reagent and 1 mL saturated sodium carbonate solution. Absorption was measured at 725 nm to obtain the calibration curve. Finally, the total concentration of polyphenol in the olive oil samples was determined as ppm of Gallic acid.

Determination of FAAEs

For the determination of FAAEs, the European Official Methods of Analysis, suggesting a GC assay, was used.[19] Accordingly, a 100-mg olive oil sample was dissolved in 10 mL n-hexane in a 20-mL test tube and 100 µL of 2 N potassium hydroxide in methanol was added. The prepared sample solution was vortexed for 30 seconds and centrifuged for 15 min. Afterwards, the supernatant phase was transferred into a 2-mL autosampler vial for chromatographic analysis.

Chromatographic analyses were performed on an Agilent 6890 GC (Agilent Technologies, Santa Clara, USA) fitted with an FID detector. The column used was a capillary HP-88 J&W 112-88A7 (length 100 m, id 0.25 mm and film thickness 0.2 µm). The operating conditions were as follows: the inlet temperature was 250°C; injection volume was 2 µL; the carrier gas was helium with a flow rate of 2 mL/min and 1:50 split ratio; oven temperature was set to 120°C for 1min initially, and then it was first increased up to 175°C (i.e., 10°C/min rate), then increased to 220°C (i.e., 3°C/min rate), where it was maintained for 5 min; the detector temperature was set to 280°C.

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 software package (SPSS Inc., Chicago, IL, USA). Mean scores and standard deviations were calculated with respect to the assay results practiced in triplicate. The paired-samples t-test was employed in order to show the statistical significance between the mean scores of the 3 and 6 months samples.

RESULTS AND DISCUSSION

In order to determine the basic quality indices of the samples collected, the percent free acid, PVs, and the specific absorption coefficients were measured first. The results obtained for the percent free acid of the samples and the reference are shown in Figure 1. The IOOC defines and designates the classification of olive oil mainly according to their FFA content.[20] Accordingly, none of the three month samples can be classified as extra virgin olive oil. Each was categorized as virgin olive oil because the results for the free acid content for each 3 month sample was found less than 2 grams per 100 grams oil. With respect to the FFA content in the 6 month samples, the categorization as virgin olive oil was saved for the majority of the samples, although 2 of them (i.e., 5O, and 6O, both of which were cold procedure products) appeared to be ordinary virgin olive oil according to the IOOC guidelines. Furthermore, the reference commercial sample was shown to keep its extra virgin olive oil property in the first 6 months following its production. It is noteworthy that the increase in percent FFA content in each sample and the reference was found statistically significant (i.e., $p<0.05$).

One of the major parameters that shows the quality of olive oil is the PV, defined as the measure of total peroxides in olive oil expressed as milliequivalent of O_2/kg oil. The upper standard for the peroxide is 20 meq/kg oil.[21,22] Besides sample 11F, each sample was shown to possess peroxide levels less than the upper standard in the 3rd month analysis (i.e., Figure 2). However, there was a serious increase in terms of PV, an indication of oxidation in all of the samples tested. Indeed, more than half of the samples tested (1O, 2O, 4O, 5O, 6O, 7O, 10O, and 11O) were shown to have peroxide levels less than 20 meq/kg oil in their 6 month analysis. Besides sample 3O, all of the cold procedure products were found to possess a higher tendency for oxidation. The increase in the PV for the reference sample was also established; however, it did not reach the upper standard peroxide level (i.e., 20 meq/kg oil), even at the 6th month. Moreover, the increase observed for the PV of each sample, including the reference, was found statistically significant (i.e., $p<0.05$). The oxidation status of each sample was also analyzed in other experiments.

The measurement of absorptions at 232 nm (i.e., K_{232}) and 270 nm (i.e., K_{270}) are important parameters for the estimation of the oxidation stage of olive oil. The increase in the number of conjugated diene and trienes contribute to K_{232}, and the secondary oxidation resulting in the formation of aldehydes and ketones is effective for K_{270}.[23] The European Regulation standard limit value for olive oil expresses K_{232} ≤2.5 for extra virgin olive oil and K_{232} ≤2.6 for both virgin olive oil and ordinary olive oil. On the other hand, K_{270} values are restricted to ≤0.2 for extra virgin olive oil and ≤0.25 for both virgin olive oil and ordinary olive oil.[24] As seen in Figure 3, all of the samples, regardless of their analysis time and extraction procedure, had K_{232} levels less than 2.5. However, it is obvious that there was an increase in K_{232} levels from the 3rd to 6th month samples, making the K_{232} levels become closer to the upper limit of 2.5, and 2.6 for the extra virgin olive oil, virgin olive oil, and ordinary olive oil, respectively. On the other hand, the K_{270} measurements in the 3 month samples classified almost all of the samples (besides sample 9F) under the extra virgin olive oil quality (i.e., Figure 4). However, similar to the observation obtained for the K_{232} values, all of the samples tested in their 6th month obviously indicated an increase, all above 0.25. This is an absolute indication of oxidation as determined in UV studies (i.e., K_{232} and K_{270} measurements) concomitant to the results obtained for the peroxide measurements. Besides the K_{232} value change for the samples 2 (i.e., 2F and 2O), and 4 (i.e., 4F and 4O) and K_{270} value changes for the samples 5 (i.e., 5F and 5O), and 9 (i.e., 9F and 9O), and the reference, all changes for the rest of the samples were found to be statistically significant (i.e., $p<0.05$).

Phenolic compounds are also present in olive oil. They are important for biologic systems with respect to their antioxidant capacity and have significant parameters that show the level of

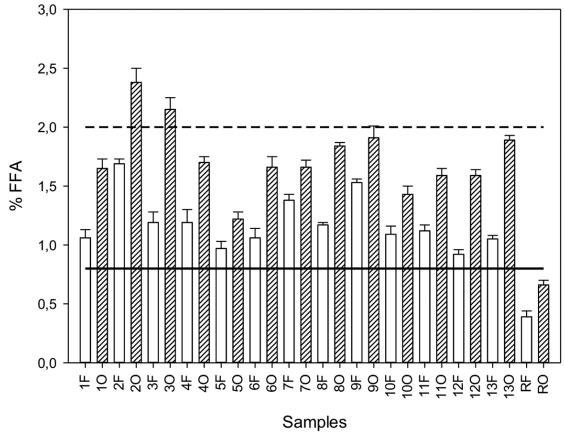

Figure 1. Percent free fatty acid of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid and the dashed lines indicate the highest levels for the free acid content of the extra virgin olive oil, and the virgin olive oil, respectively

FFA: Free fatty acid

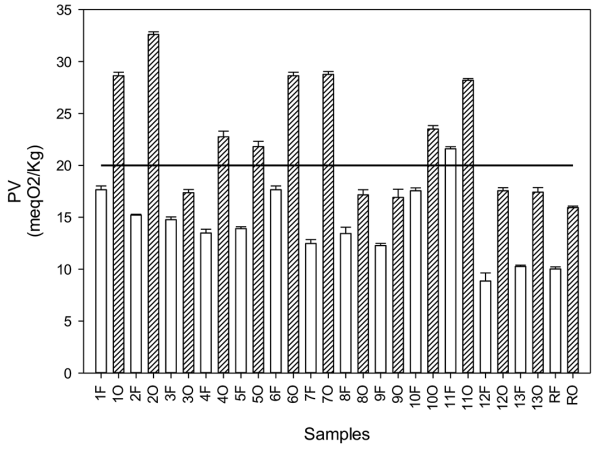

Figure 2. Peroxide value of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line indicates the upper standard for the peroxide level PV: Peroxide value

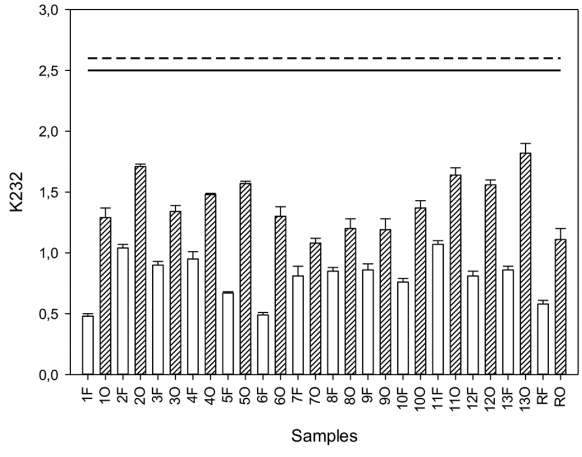

Figure 3. K^2_{32} level of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line, and the dashed lines indicate the upper standards respectively for the extra virgin olive oil, and virgin olive oil

oxidation in olive oil.[25,26] According to the results we obtained for the TPCs of the samples (i.e., Figure 5), it was obvious that the TPC of the samples were quite low in comparison with the industrially produced reference olive oil product in both 3rd and 6th month analyses. Furthermore, hot and cold extraction techniques made no difference in terms of the presence of phenolic compounds because both techniques' products had a TPC ranging around 70 ppm to a very low 10 ppm. This implies that the missing control systems in the production of traditionally produced olive oils (e.g., high exposure to light and air) and the inadequacy of the packaging resulted in oxidation of phenolic compounds. This is totally consistent with previous results obtained in previous experiments (i.e., PV values, K232, and K270 measurements) displaying the high exposure of samples to oxidation. It is also clear that the decrease in TPC was also found statistically significant for each sample, including the reference (i.e., $p<0.05$). In contrast to the regional belief of the public, this status also questions the nutritional level of the olive oil produced under primitive conditions without the presence of industrialized systems. The percent FFA, peroxide, and K_{232} and K_{270} measurements of the samples and the reference concomitant to statistical analyses are provided in detail in Table 2.

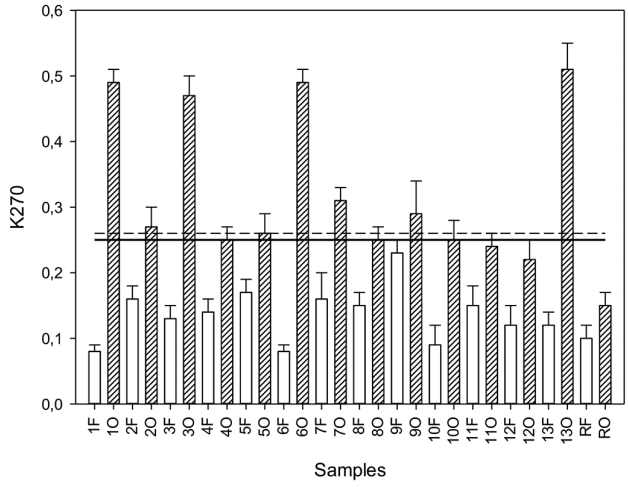

Figure 4. K_{270} level of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line, and the dashed lines indicate the upper standards respectively for the extra virgin olive oil, and virgin olive oil

Table 1. The origin, extraction type, and the coding of the olive oil samples employed

Code	Place of origin	Extraction type
1	Bostanci (Zodia)	Cold
2	Camlikoy (Camlikoy)	Cold
3	Yesilirmak (Limnidi)	Cold
4	Yesilirmak (Limnidi)	Cold
5	Guzelyurt (Morphou)	Cold
6	Guzelyurt (Morphou)	Cold
7	Yedidalga (Potamos du Gambo)	Hot
8	Yedidalga (Potamos du Gambo)	Hot
9	Yedidalga (Potamos du Gambo)	Hot
10	Yesilirmak (Limnidi)	Hot
11	Yesilyurt (Pentagia)	Hot
12	Yesilyurt (Pentagia)	Hot
13	Yesilyurt (Pentagia)	Hot

Besides their function for coloration, pigment contents (i.e., chlorophylls and carotenoids) present in olive oil are critical for the stability of the oil and for their antioxidant activity.[27] Therefore, the change in the levels of these compounds is another indication to measure the level of oxidation in olive oil samples. As shown in Figure 6, each sample tested in both the 3rd and 6th months was found to possess a lower chlorophyll amount in comparison with the reference olive oil. On the other hand, the carotenoid levels were also found lower in comparison with the reference product (i.e., Figure 7). In general, chlorophyll and carotenoid levels are expected to be around the 1-3 ppm range.[28] Therefore, the levels obtained for the sample olive oils definitely showed their lower content in terms of these pigment contents. Besides samples 7, 8, 13, and the reference, the changes in chlorophyll and the carotenoid levels were all found statistically insignificant (i.e., $p>0.05$). Similar to the results obtained for the TPC of the samples analyzed, this status implies inadequacy in both production conditions and packaging systems, thus making the olive oil products highly susceptible to oxidation. The measurements in total phenol and pigment content of the samples concomitant to statistical analyses are provided in detail in Table 3.

The composition of the fatty acids in the samples tested was measured using a GC method. Table 4 represents the results obtained for the six major fatty acids considered in this study

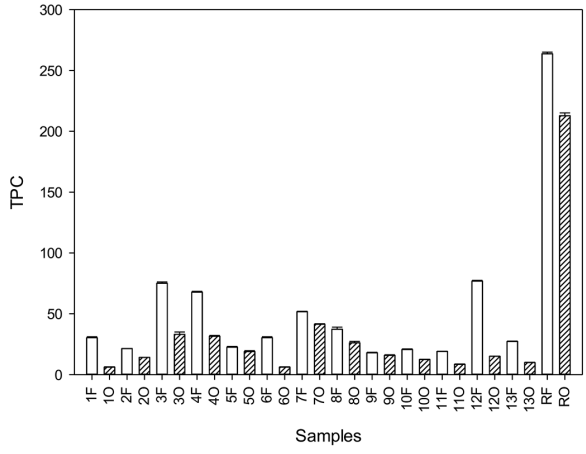

Figure 5. Total phenol content of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively

TPC: Total phenol content

Table 2. Percent free fatty acid, peroxide, and K_{232}, and K_{270} measurements

Samples	FFA %	p (FFA)	PV (meqO_2/Kg)	p (PV)	K_{232}	p (K_{232})	K_{270}	p (K_{270})
1F	1.06±0.07	**0.009***	17.66±0.36	**0.001***	0.48±0.02	**0.005***	0.08±0.01	**0.002***
1O	1.65±0.08		28.63±0.34		1.29±0.08		0.49±0.02	
2F	1.69±0.04	**0.008***	15.21±0.08	**<0.001***	1.04±0.03	**0.002***	0.16±0.02	**0.053***
2O	2.38±0.12		32.60±0.26		1.71±0.02		0.27±0.03	
3F	1.19±0.09	**0.002***	14.76±0.27	**0.004***	0.90±0.03	**0.001***	0.13±0.02	**0.002***
3O	2.15±0.10		17.37±0.31		1.34±0.05		0.47±0.03	
4F	1.19±0.11	**0.025***	13.48±0.36	**0.003***	0.95±0.06	**0.003***	0.14±0.02	**0.003***
4O	1.70±0.05		22.76±0.54		1.48±0.01		0.25±0.02	
5F	0.97±0.06	**0.011***	13.90±0.18	**0.001***	0.67±0.01	**<0.001***	0.17±0.02	0.057
5O	1.22±0.06		21.81±0.51		1.57±0.02		0.26±0.03	
6F	1.06±0.08	**0.009***	17.66±0.36	**0.001***	0.49±0.02	**0.005***	0.08±0.01	**0.002***
6O	1.66±0.09		28.63±0.34		1.30±0.08		0.49±0.02	
7F	1.38±0.05	**0.009***	12.47±0.38	**<0.001***	0.81±0.08	0.054	0.16±0.04	**0.004***
7O	1.66±0.06		28.76±0.28		1.08±0.04		0.31±0.02	
8F	1.17±0.02	**0.001***	13.43±0.61	**0.028***	0.85±0.03	**0.008***	0.15±0.02	**0.016***
8O	1.84±0.03		17.16±0.50		1.20±0.08		0.25±0.02	
9F	1.53±0.03	**0.010***	12.27±0.21	**0.013***	0.86±0.05	0.054	0.23±0.02	0.146
9O	1.91±0.10		16.92±0.79		1.19±0.09		0.29±0.05	
10F	1.09±0.07	**0.003***	17.56±0.28	**0.003***	0.76±0.03	**0.003***	0.09±0.03	**0.004***
10O	1.43±0.07		23.50±0.33		1.37±0.06		0.25±0.03	
11F	1.12±0.05	**0.001***	21.60±0.20	**<0.001***	1.07±0.03	**0.002***	0.15±0.03	**0.028***
11O	1.59±0.06		28.20±0.17		1.64±0.06		0.24±0.02	
12F	0.92±0.04	**0.001***	8.96±0.78	**0.005***	0.81±0.04	0.063	0.12±0.03	**<0.001***
12O	1.59±0.05		17.56±0.30		1.56±0.04		0.22±0.03	
13F	1.05±0.03	**0.001***	10.26±0.13	**0.002***	**0.86±0.03**	**0.003***	**0.12±0.02**	**0.002***
13O	1.89±0.04		17.42±0.45		**1.82±0.08**		**0.51±0.04**	
Reference F	0.39±0.05	**0.027***	10.02±0.20	**0.000***	0.58±0.03	**0.014***	0.10±0.02	0.138
Reference O	0.66±0.04		15.94±0.15		1.11±0.09		0.15±0.02	

F, O, and R represent 3 months, 6 months, and reference samples, respectively.
*: significant if $p<0.05$

[i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), and linoleic acid (C18:2), and linolenic acid (C18:3)]. First, it is noteworthy that there was a decrease in the fatty acid composition of the samples analyzed from the three to six month analyses, regardless of the fatty acid type. The percentage of oleic acid content of olive oil samples varied from 30 to 46% both at their 3rd and 6th month analyses (Figure 8). This indicates that none of the samples could be classified as extra virgin olive oil because the IOOC confirms 55-83% olive oil presence in extra virgin olive oils.[20] Furthermore, the analysis of other fatty acids also indicated that their ratio was at the lower limits of appreciable amounts, as required by the IOCC. The presence of oxidation as proven via several methods might clearly explain the loss of fatty acids in the samples to oxidation to other ingredients such as polyenes in the first state, and aldehydes and ketone in the second stage. In contrast to the samples, the reference product was shown to possess extra virgin olive oil quality in both the 3rd and 6th month analyses. Besides the change for palmitic acid (C16:0), the rest of the changes for each fatty acid analyzed was found insignificant (i.e., $p>0.05$).

Table 3. Total phenol and pigment content

Samples	TPC (ppm)	p (TPC)	Chlorophyll (ppm)	p (Chlp)	Carotenoids (ppm)	p (Car)
1F	30.27±0.81	**<0.001***	0.14±0.05	0.074	1.27±0.06	0.530
1O	6.16±0.25		0.14±0.03		1.20±0.20	
2F	21.40±0.10	**<0.001***	0.11±0.01	0.423	0.99±0.08	0.131
2O	14.13±0.06		0.10±0.01		0.91±0.03	
3F	75.03±1.14	**0.002***	0.14±0.01	0.122	1.08±0.13	0.236
3O	33.03±1.97		0.10±0.02		0.97±0.17	
4F	67.77±0.65	**<0.001***	0.13±0.01	0.225	0.90±0.00	0.339
4O	31.57±0.61		0.12±0.01		0.86±0.06	
5F	22.53±0.67	**0.003***	0.15±0.02	0.213	0.86±0.06	0.093
5O	18.90±0.82		0.11±0.03		0.82±0.04	
6F	30.27±0.81	**<0.001***	0.14±0.05	0.742	1.27±0.06	0.529
6O	6.16±0.25		0.14±0.03		1.20±0.20	
7F	51.70±0.36	**0.002***	0.13±0.04	0.173	1.17±0.06	**0.020***
7O	41.33±0.45		0.09±0.03		0.93±0.06	
8F	37.30±1.71	**0.013***	0.15±0.01	**0.010***	1.10±0.17	0.529
8O	26.13±1.16		0.11±0.01		1.03±0.06	
9F	17.87±0.49	0.051	0.13±0.03	0.576	1.17±0.06	0.199
9O	15.77±0.49		0.11±0.04		1.00±0.10	
10F	20.60±0.53	**0.001***	0.15±0.02	0.474	1.02±0.04	0.303
10O	12.40±0.20		0.12±0.05		0.98±0.02	
11F	18.97±0.25	**0.001***	0.10±0.02	0.860	1.13±0.06	0.055
11O	8.43±0.31		0.11±0.01		0.96±0.04	
12F	76.83±0.64	**<0.001***	0.12±0.03	0.383	1.20±0.10	0.067
12O	15.00±0.10		0.09±0.02		0.94±0.04	
13F	27.20±0.36	**<0.001***	0.11±0.01	0.095	1.17±0.02	**0.016***
13O	9.90±0.20		0.08±0.01		0.83±0.06	
Reference F	263.73±1.35	**0.001***	1.08±0.02	**0.009***	2.53±0.04	**0.014***
Reference O	212.83±2.32		1.02±0.03		2.48±0.03	

F, O, and R represent 3 months, 6 months, and reference samples, respectively.
*: significant if $p<0.05$

CONCLUSION

Although olive oil production and consumption is very popular in Northern Cyprus, the quality indices of the oil prepared via traditional methods were found to be low with respect to the results of this study. Indeed, in almost all tests employed, exposure to oxidation was quite unique to these products regardless of the extraction procedure employed (i.e., hot and cold extractions). This absolutely implies inadequacy in production techniques, which may be mainly attributed to insufficient protection against light and air during the production process. Furthermore, the deficiencies regarding the packaging of the oil are another drawback to limit the shelf-life of these products.

This study, known to be conducted for the first time in Northern Cyprus, has highlighted the high oxidation exposure of olive oil produced within the country which employs traditional techniques. Therefore, the results will contribute to the awareness of both producers and consumers. In particular, it will have a significant effect on producers to change their production methods in terms of paying attention to control

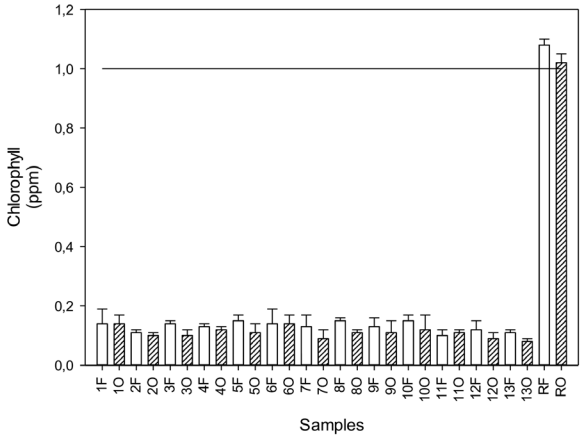

Figure 6. Chlorophyll content of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line indicates the expected lowest standard for the extra virgin olive oil

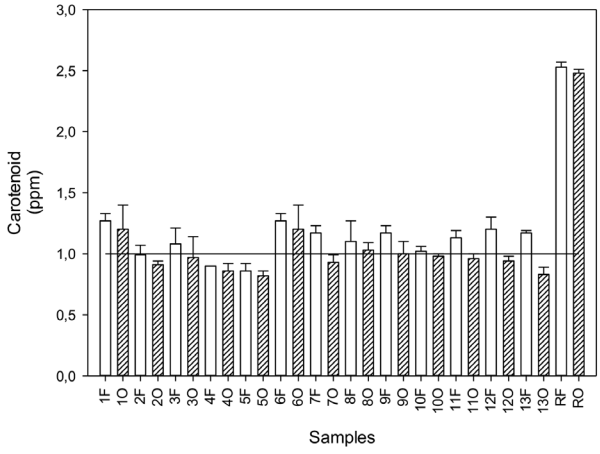

Figure 7. Carotenoid content of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line indicates the expected lowest standard for the extra virgin olive oil

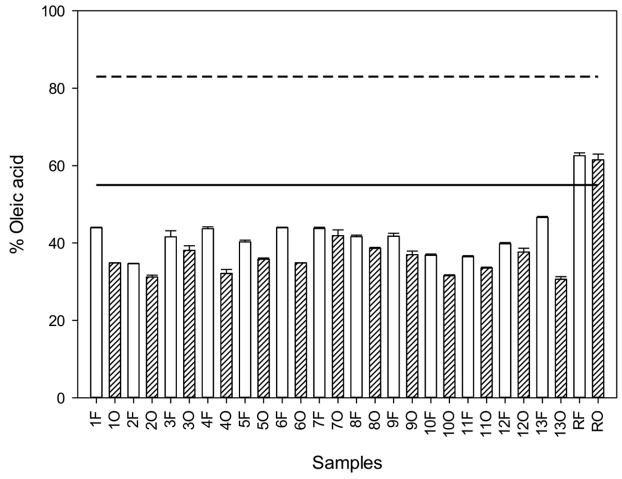

Figure 8. Percent oleic acid of the samples and reference

F, O, and R represent 3 months, 6 months, and reference samples, respectively. The solid line, and the dashed lines indicate the standard range for the extra virgin olive oil

Table 4. Fatty acids composition of the samples and the reference

Samples	C16:0 (%)	p C16:0	C16:1 (%)	p C16:1	C18:0 (%)	p C18:0	C18:1 (%)	p C18:1	C18:2 (%)	p C18:2	C18:3 (%)	p C18:3
1F	6.79±0.17	0.071	0.39±0.02	**0.011***	1.57±0.02	**<0.001***	43.94±0.13	**<0.001***	5.56±0.06	**<0.001***	0.18±0.01	0.130
10	6.02±0.21		0.27±0.04		1.33±0.02		34.83±0.04		3.92±0.06		0.16±0.02	
2F	7.87±0.06	**<0.001***	0.33±0.01	**0.014***	2.29±0.04	**0.023***	34.63±0.10	**0.007***	7. 52±0.18	0.255	0.22±0.02	**0.017***
20	5.96±0.06		0.26±0.02		1.75±0.10		31.21±0.46		7.14±0.26		0.15±0.03	
3F	7.22±0.03	**0.038***	0.35±0.02	**0.037***	2.49±0.08	**0.022***	41.58±1.55	0.156	6.26±0.10	**0.004***	0.26±0.02	0.078
30	7.02±0.10		0.21±0.04		1.99±0.06		38.07±1.20		5.25±0.04		0.19±0.02	
4F	10.24±0.10	**0.010***	0.59±0.03	**0.032***	2.38±0.04	**0.015***	43.67±0.49	**0.002***	7.61±0.31	**0.002***	0.32±0.02	**0.001***
40	7.69±0.45		0.38±0.04		1.90±0.10		32.10±1.04		5.02±0.19		0.15±0.01	
5F	8.50±0.11	**0.003***	0.44±0.04	0.059	2.05±0.08	0.176	40.27±0.45	**0.005***	7.82±0.21	**0.026***	0.25±0.02	0.324
50	7.41±0.19		0.32±0.03		1.89±0.19		35.79±0.31		6.97±0.22		0.22±0.03	
6F	6.79±0.17	0.071	0.39±0.02	**0.011***	1.57±0.02	**<0.001***	43.94±0.13	**<0.001***	5.56±0.06	**<0.001***	0.18±0.01	0.130
60	6.02±0.21		0.27±0.04		1.33±0.02		34.83±0.04		3.92±0.06		0.16±0.02	
7F	10.06±0.14	**0.045***	0.76±0.05	**0.015***	2.51±0.09	**0.001***	43.73±0.35	0.178	8.34±0.29	**0.001***	0.31±0.02	0.058
70	9.13±0.47		0.51±0.03		1.59±0.08		41.86±1.50		4.93±0.16		0.21±0.05	
8F	9.50±0.10	0.065	0.54±0.07	**0.019***	2.47±0.04	**0.006***	41.63±0.38	**0.015***	7.57±0.42	0.280	0.29±0.03	0.069
80	8.30±0.46		0.40±0.04		2.06±0.08		38.60±0.26		6.84±0.57		0.25±0.03	
9F	8.97±0.15	0.069	0.67±0.04	**0.046***	2.22±0.11	0.083	41.73±0.76	**0.015***	7.55±0.05	**0.003***	0.28±0.03	**0.034***
90	8.23±0.32		0.45±0.06		1.81±0.13		36.97±0.93		6.15±0.17		0.18±0.01	
10F	7.84±0.07	**0.001***	0.45±0.04	0.148	1.95±0.06	0.054	36.83±0.31	**0.003***	5.38±0.08	**0.001***	0.18±0.01	0.157
100	5.68±0.20		0.35±0.05		1.59±0.11		31.50±0.26		4.77±0.05		0.13±0.03	
11F	7.44±0.48	0.434	0.52±0.04	0.119	1.41±0.09	**0.026**	36.43±0.27	**<0.001***	5.61±0.07	**0.001***	0.18±0.02	0.188
110	7.13±0.08		0.37±0.08		1.30±0.11		33.53±0.21		4.76±0.04		0.15±0.01	
12F	6.90±0.19	**0.006***	0.39±0.04	**0.012***	1.65±0.08	0.148	39.77±0.31	0.067	4.46±0.09	**0.003***	0.18±0.02	0.192
120	6.01±0.11		0.30±0.03		1.52±0.05		37.63±0.99		3.33±0.12		0.15±0.01	
13F	9.92±0.30	**0.005***	0.77±0.05	**0.007***	2.16±0.06	**0.023***	46.60±0.26	**0.001***	6.48±0.07	**0.001***	0.28±0.01	**0.006***
130	8.33±0.46		0.49±0.06		1.80±0.04		30.60±0.70		4.40±0.11		0.16±0.01	
Reference F	9.28±0.03	**0.015***	1.18±0.01	0.115	3.44±0.05	0.313	62.57±0.72	0.457	8.89±0.02	0.097	0.50±0.04	0.742
Reference O	9.20±0.03		1.11±0.06		3.37±0.06		61.50±1.48		8.75±0.06		0.52±0.06	

systems (e.g., harvesting and production periods, packaging, and appropriate labeling) to prevent the continuous oxidation in the olive oil produced.

ACKNOWLEDGEMENTS

This work was supported by Faculty of Pharmacy, Eastern Mediterranean University, research funds.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):200-206
DOI: 10.4274/tjps.46320

ORIGINAL ARTICLE

Genotype and Allele Frequency of *CYP3A4 -392A>G* in Turkish Patients with Major Depressive Disorder

Majör Depresif Bozukluğu Olan Türk Hastalarında *CYP3A4 -392A>G* Genotip ve Allel Frekansı

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ABSTRACT

Objectives: Genetic polymorphisms may help for individualized drug dosing and improved therapeutics. CYP3A4 is responsible for the metabolism of more than 50% of the commonly used drugs and metabolizes typical antipsychotic medications and antidepressant drugs. The objective of the study was to assess the genotype and allele frequencies of *CYP3A4 -392A>G* in Turkish patients with major depressive disorder receiving any SSRIs and to compare these results with the frequencies of other ethnic groups.

Materials and Methods: Genotyping analyses of *CYP3A4 -392A>G* was conducted on 84 Turkish patients using the PCR-RFLP technique.

Results: The allele frequencies were found as 0.982 (A) and 0.018 (G) for *CYP3A4 -392A>G*. The genotype frequencies were determined as 0.976 (AA), 0.012 (AG), and 0.012 (GG). The genotype frequencies were consistent with the Hardy-Weinberg equilibrium.

Conclusion: The genotype and allele frequencies of *CYP3A4 -392A>G* were determined to be low in Turkish patients with major depressive disorder receiving SSRIs. Furthermore, the results of the study were compared with those of other ethnic groups and they displayed pronounced differences among other ethnic groups, especially black subjects.

Key words: *CYP3A4 -392A>G*, polymorphism, Turkish patients, major depressive disorder

ÖZ

Amaç: Genetik polimorfizmler, bireyselleştirilmiş ilaç dozlaması ve geliştirilmiş terapötikler için yardımcı olabilir. CYP3A4 yaygın olarak kullanılan ilaçların %50'sinden fazlasının metabolizmasından sorumludur ve tipik olarak antipsikotik ilaçlar, antidepresan ilaçları metabolize eder. Bu çalışmanın amacı, herhangi bir SSGİ alan majör depresif bozukluğu olan Türk hastalarında *CYP3A4 -392A>G*'nin genotip ve alel frekanslarını değerlendirmek ve sonuçlarımızı diğer etnik gruplardaki frekanslarla karşılaştırmaktır.

Gereç ve Yöntemler: *CYP3A4 -392A>G*'nin genotiplendirme analizi, 84 Türk hastasında PZR-RFLP tekniği ile gerçekleştirilmiştir.

Bulgular: *CYP3A4 -392A>G* için allel frekanslarının 0.982 (A) ve 0.018 (G) olduğu saptanmıştır. Genotip frekanslarının ise 0.976 (AA), 0.012 (AG) ve 0.012 (GG) olduğu tespit edilmiştir. Genotip frekansları Hardy-Weinberg dengesiyle uyumludur.

Sonuç: *CYP3A4 -392A>G*'nin düşük frekansı, *CYP3A4* ilaç metabolize edici enzimin SSGİ'ler üzerinde oldukça düşük bir etkisinin olacağı önerilmektedir. Bunun yanı sıra, araştırmanın sonuçları diğer etnik gruplarla karşılaştırılmış olup etnik grup farklılıklarının özellikle de siyah deneklerde belirlenmiştir.

Anahtar kelimeler: *CYP3A4 -392A>G*, polimorfizm, Türk hastalar, majör depresif bozukluk

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Received: 12.05.2017, **Accepted:** 19.07.2017

INTRODUCTION

Cytochrome P450 (CYP) is the major metabolizing enzymatic system in humans and CYP enzymes are responsible for the metabolism of exogenous compounds, including most clinically used drugs, mutagens, carcinogens,[1,2] and some endogenous compounds, such as prostaglandins, steroids, vitamins, fatty acid derivatives and retinoic acid derivatives, and thromboxanes.[2,3] CYP enzymes are responsible for the biotransformation of lipophilic compounds to polar metabolites, which can be excreted by the urine or bile. There are three major CYP families that encode enzymes that play an important role in phase I metabolism: CYP1, CYP2, and CYP3.[3] The CYP3A subfamily is the most abundant CYP enzyme and represents about 30% of the total CYP in the human liver.[2] Approximately 65% of current drugs used are metabolized by CYP enzymes and 45-60% of clinically administered drugs, and exogenous and endogenous compounds such as streoids, are metabolized by the CYP3A subfamily.[4,5] The CYP3A subfamily consists of 4 members: *CYP3A4*, *CYP3A5*, *CYP3A7*, and *CYP3A43*.[5] The *CYP3A4* enzyme is the most abundant CYP isoform in the liver and intestine, representing 60% and 70% of the total P450 amount, respectively. *CYP3A4* is responsible for the metabolism of more than 50% of commonly prescribed drugs and metabolizes typical antipsychotic medications, antidepressant drugs (Table 1).[6] Its interindividual hepatic expression varies 60-fold, resulting in therapeutic failure, unpredictable adverse effects or severe drug toxicity.[7]

The *CYP3A4* gene is located on chromosome 7q21.3-q22.1, is 27,592 base pairs (bp) long, and has 13 exons.[3,8] Genetic polymorphisms of *CYP3A4* were unknown until 1996.[8] However, nowadays, *CYP3A4* is known to be polymorphic, and more than 30 single nucleotide polymorphisms have been described in the *CYP3A4* gene. The most common single-nucleotide polymorphism *-392A>G* in the promoter region of the *CYP3A4* gene has been described. *CYP3A4 -392A>G* (rs2740574) is also known as *CYP3A4*1B*. It is known that the *CYP3A4*1B* polymorphism alters the transcription efficiency of the gene and hence the overall activity of *CYP3A4*.[9]

Selective serotonin reuptake inhibitors (SSRIs) are the first-line treatment for mild-to-severe major depressive disorder (MDD).[10] The objective of this study was to assess the genotypic and allelic frequencies of the *CYP3A4*1B* in Turkish patients with MDD receiving SSRIs and to compare the results with frequencies in other ethnic groups.

MATERIALS AND METHODS

Subjects

The study was conducted on 84 Turkish patients with MDD at the Departments of Psychiatry, Schools of Medicine, Ankara University and Kırıkkale University, Turkey. All participants were administered with SSRIs. Approval for this study was obtained from the Ethics Committee of the Ankara University (21 April 2008, protocol no: 128-3581). The study was conducted in accordance with Good Clinical Practices and the Helsinki Declaration. All subjects gave their written informed consent to participate in this study. The demographic data of the patients with MDD are shown in Table 2.

Blood sampling

Blood samples (10 mL) were collected in vacutainer tubes containing EDTA as an anticoagulant between 08:00 and 09:00 a.m. at the 4th and/or 6th weeks of treatment. The Wizard Genomic DNA Purification Kit (Promega) was used to isolate genomic DNA from the cell fraction. DNA yields were determined by measuring the absorbance at 260 nm (A_{260}). All samples were stored at -80˚C until analysis.

Genotyping

The *CYP3A4*1B* (rs 2740574; *-392A>G*) polymorphism was identified using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method of Cavalli et al.[11] with minor modifications. The primers employed were F: 5'-GGAATGAGGACAGCCATAGAGACAAGGGGA-3', R: 5'CCTTTCAGCTCTGTGT TGCTCTTTGCTG-3'. PCR was performed in a 25-µL reaction mixture containig 300-500 ng of genomic DNA, 10 pmol of each primer, 0.2 mM each deoxynucleotidetriphosphate, 10 x PCR buffer, 1.5 mM $MgCl_2$, and 1.25 unit of Taq polymerase (Fermentase) on the MBS Satellite Thermal Cycler (Thermo, UK). After initial denaturation for 5 min at 97˚C, PCR was performed for 30 cycles of 60 s at 95˚C, 90 s at 60˚C, 60 s at 72˚C, and with a final step of 72˚C for 10 min for elongation. No added DNA (negative control) reactions were included in each PCR analysis to ensure that the agents

Table 1. Common drugs metabolized by *CYP3A4*[6]

Group of drugs	Drug name
Antidepressants (SSRIs; SNRIs; tricyclics; others)	Citalopram, escitalopram, paroxetine, fluoxetine; venalafaxine, trazodone; amitriptyline, imipramine, clomipramine; buspirone nefazodone, mirtazapine
Antipsychotics (first generations; second generations)	Haloperidol, perphenazine; aripiprazole, quetiapine, risperidone, ziprasidone
Benzodiazepines	Alprazolam, diazepam, medazolam, temazepam, lorazepam, clonazepam
Opiates	Codeine, methadone, fentanyl, buprenorphine
Hypnotics	Zopiclone, zaleplon, zolpidem
Antibiotics	Erythromycin, clarithromycin, telithromycin
Phosphodiesters inhibitors	Sildenafil, tadalafil

contained no contaminating DNA. The PCR product (385 bp) was analyzed electrophoretically on a 2% agarose gel stained with ethidium bromide (500 ng/mL). Ten microliters of the PCR product were digested at 37˚C overnight with 10 U of *MboII* with the appropriate buffer in a total volume of 20 µL. As shown in Figure 1, the digestion resulted in fragments of 175, 169, and 41 bp for the AA (wild type), and fragments of 210 and 175 bp for the GG (mutant). The digested fragments were electrophoresed on a 2% agarose gel and visualized using ethidium bromide.

Table 2. Baseline characteristics of the patients with major depressive disorder

Demographic and genotypic characteristics	Mean ± SD	Range (min-max)
Body weight (kg)	70.12±14.39	45.5-105
BMI (kg/m^2)	25.94±5.14	16.1-41.14
	n	%
Sex		
Female	68	81
Male	16	19
Age range		
≤40	53	**63**
>40	31	**37**
***CYP3A4* genotypes**		
Genotypic frequencies		
AA (or **1A*1A)*	82	97.6
AG (or **1A*1B)*	1	1.2
GG (or **1B*1B)*	1	1.2
Allelic frequencies		
A (or **1A*)	165	98.6
G (or **1B*)	3	1.8

BMI: Body mass index

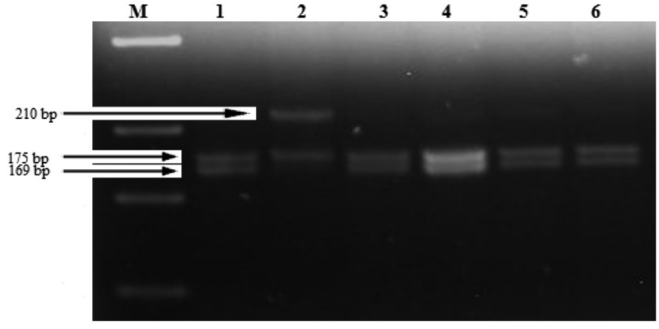

Figure 1. RFLP for the CYP3A4*1B polymorphism. Lane M: Marker, Lane 2: mutant (210, 175 bp), Lane 1,3-6: wild type (175, 169, 41 bp)

Statistical analysis

Genotype counting was used to calculate the allele and genotype frequencies. The observed and expected genotype frequencies of *CYP3A4* were compared using the Hardy-Weinberg equilibrium. The comparison of the allele frequencies in the present investigation with those in other populations was made using the chi-square test. *P* values <0.05 and <0.001 were considered statistically significant.

RESULTS

*CYP3A4*1B* (-392A>G) polymorphism analysis was conducted on 84 Turkish patients with MDD. Of the 84 patients, 68 (81% of patients) were female, whereas 16 (19% of them) were male (*p>0.05*) (Table 2). The body weight of the patients varied from 45.5 to 105 kg, with a mean of 70.12±14.39 kg. The body mass index (of the patients) ranged from 16.1 to 41.14 kg/m^2, with a mean of 25.94±5.14 kg/m^2. In the study, 53 subjects (63%) were aged ≤40 years, and 31 subjects (37%) were aged >40 years.

The frequencies of the *AA*, *AG*, and *GG* genotypes were 0.976, 0.012, and 0.012, respectively. According to these results, the frequencies of *A* and *G* alleles were 0.982 and 0.018, respectively (Table 2). These results were consistent with the expected genotype frequencies of the Hardy-Weinberg equilibrium (*p>0.05*).

DISCUSSION

Factors that can influence the response of a patient to any given drug depend on intrinsic (e.g., genetic and non-genetic factors such as sex, age, organ dysfunctions, disease state, and race/ethnicity) and extrinsic factors (e.g., use of alcohol, smoking, diet, and concomitant medication).[12,13] Genetics is estimated to account for 20 to 95% of variability in drug effects and disposition.[14] It has been shown that much of this variability is produced by genetic polymorphisms of the CYP enzymes.[4] CYP enzymes perform extensive structural differences because of genetic polymorphisms in the corresponding genes, and thus causing different enzymatic activities and giving rise to great intra- and inter-population variation in drug efficacy and adverse reactions.[15]

Approximately 65% of drugs in current use are metabolized by CYP enzymes, and 45-60% of clinically administered drugs, exogenous and endogenous compounds such as streoids, are metabolized by the *CYP3A* subfamily.[4,5] *CYP3A4* is a polymorphic enzyme, and its interindividual hepatic expression varies 60-fold.[7] *CYP3A4*1B*, described as the most common variant, has been speculated to have reduced activity.[16] Significant differences in allele frequencies of *CYP3A* variant occur among ethnic groups.[16] Polymorphisms in human xenobiotic metabolizing genes show parallelism in ethnic, racial, and geographic distribution, and the ethnic-specific impact on CYP genes is known.[9]

In this study, we aimed to investigate the *CYP3A4*1B* allele frequencies in Turkish patients with MDD receiving SSRIs and to compare the results with the frequencies of other ethnic groups. The allele frequencies in the Turkish population were

Table 3. Allele frequencies of *CYP3A4*1B* in different ethnic populations

		CYP3A4 allele frequencies			
Population	**Healthy and control populations**	**n**	****1A***	****1B***	**References**
White					
Turkish	Healthy	186	0.986	0.014	Sayitoglu et al.[16]
Turkish	Major depressive disorder	84	0.982	0.018	The present study
Turkish	Familial Mediterranean fever patients	46	0.967	0.033	Dogruer et al.[17]
Turkish	Children with lower urinary tract symptoms	34	0.956	0.044	Gurocak et al.[18]
Turkish	Healthy children	42	0.939	0.061	Gurocak et al. [18]
Caucasian (Germany)	Hospital controls	428	0.972	0.028	Dally et al.[20]
Australian	Control for ovarian cancer	276	0.969	0.031	Spurdle et al.[21]
Australia	Control for breast cancer	500	0.967	0.033	Spurdle et al. [21]
European	Healthy	93	0.962	0.038	Garsa et al.[22]
Caucasian American (Southern California)	Healthy	117	0.961	0.039	Paris et al. [23]
Finnish	Healthy	118	0.958	0.042	Sata et al.[24]
Spanish	Healthy	163	0.957	0.043	Gervasini et al.[25]
Portuguese	Control	337	0.951	0.049	Nogal et al.[3]
Dutch Caucasian	Healthy	199	0.947	0.053	van Schaik et al.[26]
Scottish	Healthy	101	0.946	0.054	Tayeb et al.[27]
Caucasian American* (Philadelphia)	Controls	340	0.921	0.079	Zeigler-Johnson et al.[28]
Saudi*	Healthy	101	0.910	0.090	Tayeb et al.[27]
Caucasian American* (Philadelphia)	Healthy	94	0.904	0.096	Rebbeck et al.[29]
European-Brazilians*	Healthy	91	0.901	0.099	Kohlrausch et al.[30]
Hispanic*	Controls	121	0.893	0.107	Paris et al.[23]
Asians					
Taiwanese	-	130	1.000	0.000	Walker et al.[31]
Japanese	Healthy	128	1.000	0.000	Ando et al.[32]
Japanese	Healthy	77	1.000	0.000	Ball et al.[33]
Japanese	Hospital patients	416	1.000	0.000	Fukushima-Uesaka et al.[34]
Chinese	Healthy	78	1.000	0.000	Ball et al.[33]
Chinese	Healthy	118	1.000	0.000	Sata et al.[24]
Vietnamese	Healthy	78	0.979	0.021	Veiga et al.[35]
Jordanian	Healthy	173	0.965	0.035	Yousef et al.[36]
Black**					
African-Brazilians	Healthy	86	0.616	0.384	Kohlrausch et al.[30]
African	Controls	67	0.560	0.440	McDaniel et al.[37]
African American	-	70	0.470	0.530	Walker et al.[31]

Table 3. Continue					
African American	Healthy	116	0.457	0.543	Paris et al.[23]
African American	Healthy	186	0.454	0.546	Ball et al.[33]
African American	Controls	103	0.427	0.573	Bangsi et al.[38]
African American	Controls	130	0.408	0.592	Zeigler-Johnson et al.[28]
African	Healthy	150	0.333	0.667	Sata et al.[24]
Ghanaian	Healthy	100	0.310	0.690	Tayeb et al.[27]
African American	Healthy	15	0.200	0.800	Wandel et al.[39]
Ghanaian	Controls	118	0.195	0.805	Zeigler-Johnson et al.[28]
African	Healthy	88	0.176	0.824	Garsa et al.[22]

Differences in allele frequencies were assessed by χ^2 test. n total number of subjects. Significant at *p<0.05 and **p<0.001 when compared with the present study

0.982 and 0.018 for **1A* and **1B* alleles, respectively (Table 3). A comparison of the results of this investigation with the results of the other studies is presented in Table 3. Sayitoglu et al.[16] reported that **1B* allele frequency was 0.014 in healthy Turkish subjects. Dogruer et al.[17] reported that **1B* allele frequency was 0.033 in Turkish patients with familial Mediterranean fever. Gurocak et al.[18] also reported that **1B* allele frequency was 0.044 and 0.061 for Turkish children with lower urinary tract symptoms and healthy Turkish children, respectively. The allele frequencies of these studies were not significantly different from the results of this study (*p>0.05*). However, when compared with black subjects, the allele frequency of Turkish subjects showed marked differences. The **1B* variant allele frequencies were identified more frequently in African-American, African Brazilians, African, and Ghanaian individuals when compared with Turkish subjects (*p<0.001*). Furthermore, **1B* variant allele frequencies were also reported to be higher in Caucasian American (Philadelphia), Saudi, European-Brazilians, Hispanic populations when compared with Turkish populations (*p<0.05*). The distribution of **1A* and **1B* alleles in Turkish populations was similar to those reported for Caucasians (Germany), Australian, European, Finnish, Spanish, Portuguese, Caucasians American (Southern California), Ducth Caucasian, and Scottish populations (Table 3).

The allelic frequency of *CYP3A4*1B* changes among different ethnic groups; *CYP3A4*1B* allelic frequency is dominant in black subjects with a range of 38.4 to 82.4% (Table 3). On the other hand, this polymorphism is very rare in Asian ethnic groups, including Vietnamese and Jordanian groups, ranging from 0 to 9.0%. This polymorphism is absent in East Asian populations including the Japanese, Chinese, and Taiwanese, and present in White ethnic groups with a range of 1.8 to 14.3%. Consequently, it seems that the *CYP3A4*1B* polymorphism is more frequent in White ethnic groups than in East Asian populations, and is more common in black subjects than in White ethnic groups. There is a minimal clinical effect of the *CYP3A4*1B* polymorphism on Asian ethnic groups. However, the *CYP3A4*1B* polymorphism seems to be more clinically important in black subjects.

CONCLUSION

The study introduces evidence of a low frequency of *CYP3A4*1B* allele in Turkish patients and compared this frequency with those of other ethnic groups. Given the effect of *CYP3A4* on the efficacy of drugs, the genetic backgrounds of individuals and populations are accepted as a significant factor to be considered in the recipe of individualized medicine.[19] Determining the expression of *CYP3A4* may detect drug safety and efficacy and therefore help people to use the right dose of drugs.[15] *CYP3A4*1B* should be taken into consideration in populations where the allele frequency is high. On the other hand, a larger sample size would be needed to determine the *CYP3A4*1B* polymorphism in populations where the allele frequency is low.

ACKNOWLEDGEMENTS

This work was supported by the Scientific and Technological Research Council of Turkey (Project: 109S147).

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):207-211
DOI: 10.4274/tjps.32559

ORIGINAL ARTICLE

Investigation of the Antimicrobial Susceptibility Profile, Virulence Genes, and Epidemiologic Relationship of Clinical *Salmonella* Isolates

Klinik *Salmonella* İzolatlarında Antimikrobiyal Duyarlılık Profilinin, Virülans Genlerinin ve Klonal İlişkinin Araştırılması

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ABSTRACT

Objectives: The objectives of this study were to investigate the epidemiologic relationship, prevalence of the beta-lactamase and virulence genes of clinical ampicillin-resistant *Salmonella enterica*.

Materials and Methods: *In vitro* ampicillin susceptibilities of 117 *Salmonella enterica* isolates obtained between 2011-2012 from Ege University Hospital, Bacteriology Laboratory of Medical Microbiology Department were examined using disc diffusion assays in accordance with the CLSI guidelines. The MIC levels in the ampicillin-resistant bacteria were determined using the broth microdilution method. The resistant strains were serotyped by the Public Health Institution. Epidemiologic relations of resistant strains were evaluated using ERIC-PCR. The presence of beta-lactamase genes and virulence factors were detected using PCR.

Results: The 117 *S. enterica* strains had ten isolates that were resistant to ampicillin, and the MIC range of ampicillin was found as 512-128 µg/mL. Ampicillin-resistant strains were susceptible to nalidixic acid, ciprofloxacin, cefotaxime, sulfamethoxazole/trimethoprim. Four different serotypes were identified and isolates were grouped into seven clusters. Five isolates carried bla_{TEM}, and two carried the bla_{CTX-M} gene. However, it was determined that bla_{SHV} and bla_{PER} genes did not exist in these strains. Virulence genes *invA*, *pipD*, and *sopB* were found in all isolates. *sifA*, *pefA*, and *sopE* genes were found in seven, four, and three isolates, respectively.

Conclusion: Our data suggest that the rate of ampicillin resistance in *S. enterica* isolates was 8.5% in the two year period, but this ratio was generally lower than rates abroad. bla_{CTX-M} and bla_{TEM} genes could be responsible for ampicillin resistance. The bla_{SHV} gene, which is highly prevalent in our country, was not found in any strains. *sopB* and *pipD* genes, which might be associated with beta-lactam resistance, were found in all strains. It is also noteworthy that the three isolates containing the *sopE* gene, which is associated with epidemic cases, were of the same serotypes and epidemiologic clusters.

Key words: *Salmonella enterica*, beta-lactamase, virulence factors, ERIC-PCR

ÖZ

Amaç: Bu çalışmanın amacı ampisilin dirençli klinik *Salmonella enterica* izolatlarında epidemiyolojik ilişkinin, beta-laktamaz ve virülans genlerinin araştırılmasıdır.

Gereç ve Yöntemler: Ege Üniversitesi Hastanesi, Tıbbi Mikrobiyoloji Anabilim Dalı, Bakteriyoloji Laboratuvarı'nda 2011-2012 yıllarında izole edilen *S. enterica* kökenlerinin ampisilin duyarlılıkları CLSI önerileri doğrultusunda disk difüzyon yöntemiyle değerlendirildi. Ampisilin dirençli izolatlardaki ampisilin MİK değerleri yine CLSI kriterlerine göre sıvı mikrodilüsyon yöntemiyle belirlendi. Dirençli kökenler Türkiye Halk Sağlığı Kurumu tarafından serotiplendirildi. Kökenlerin epidemiyolojik ilişkisi ERIC-PZR ile incelendi. Beta-laktamaz ve virülans genlerinin prevalansı PZR ile tespit edildi.

Presented in: *In 25th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 25-28 April 2015, Copenhagen, Denmark, was presented.*

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Received: 16.05.2017, **Accepted:** 13.07.2017

Bulgular: İzole edilen 117 *S. enterica* kökeninde 10 izolat ampisilin dirençli olarak saptandı ve bu izolatların ampisilin MİK aralığı 512-128 µg/mL olarak belirlendi. İzolatlar nalidiksik asit, siprofloksasin, sefotaksim ve sulfametoksazol/trimethoprim antibiyotiklerine duyarlı bulundu. Dört farklı serotip belirlenirken, izolatlar ERIC-PZR'ye göre 7 farklı epidemiyolojik grupta yer aldı. Kökenlerin 5 tanesinde bla_{TEM}, iki tanesinde bla_{CTX-M} geni saptandı. bla_{SHV} ve bla_{PER} genleri hiçbir izolatta saptanmadı. *invA*, *pipD*, *sopB* virülans genleri tüm kökenlerde belirlenirken, *sifA*, *pefA* and *sopE* genleri sırasıyla üç, dört ve yedi kökende belirlendi.

Sonuç: Verilerimiz, *S. enterica* izolatlarında iki yıllık dönemde ampisilin direnç oranının %8.5 olduğunu ortaya koymakla birlikte bu oranın genel olarak yurtdışındaki oranlardan düşük olduğu göze çarpmaktadır. bla_{CTX-M} ve bla_{TEM} genleri ampisilin direncinden sorumlu olabilir. Ancak ülkemizde oldukça yüksek oranda saptanan bla_{SHV} genine hiçbir izolatta rastlanmamıştır. Beta-laktam direnci ile ilişkili olabileceği düşünülen *sopB* ve *pipD* genleri tüm suşlarda bulunmuştur. Ayrıca epidemik olgularla ilişkilendirilen *sopE* genini içeren üç izolatın aynı serotipe ve epidemiyolojik sınıfa ait kökenler olması dikkat çekicidir.

Anahtar kelimeler: *Salmonella enterica*, beta-laktamaz, virülans faktörleri, ERIC-PZR

INTRODUCTION

Salmonella spp. are some of the most important agents that lead to enteritis in the world. Additionally, they can lead to more critical health problems such as bacteremia and enteric fever.[1] This group of bacteria include more than 2600 serotypes and consist of two species called *S. enterica* and *S. bongori*. *S. enterica* spp. are responsible for 99% of *Salmonella* infections and *Salmonella* Enteritidis and *Salmonella* Typhimurium are the most commonly isolated serotypes both in our country and in developed countries.[2] Virulence factors, which responsible for invasion, extraintestinal spread, and intracellular survival, are encoded by genes located in the *Salmonella* pathogenicity island (SPI). Although some pathogenicity islands seem to be preserved in *Salmonella* genus, others are unique for certain serotypes. Based on presence of SPI and SPI features, *Salmonella* serotypes differ from each other in terms of adaptation in host cells, virulence factors, and severity of infections.[3]

In most cases, resultant infections do not necessitate antibiotic treatment due to the self-limiting nature of disease. However, antibiotic treatment may be necessary for some situations such as invasive infections, advanced age, and immunosuppression.[4] In such cases, ampicillin is widely used to treat *Salmonella* infections.[5] Therefore, resistance to ampicillin has emerged and beta-lactam enzymes are primarily responsible of ampicillin resistance.[6]

The objectives of this study were to investigate beta-lactam resistance, the epidemiologic relationship, serotype distribution, and the prevalence of beta-lactamase genes, namely bla_{TEM}, bla_{PER}, bla_{CTX-M}, bla_{SHV}, and the virulence genes of clinical ampicillin-resistant *S. enterica.*

EXPERIMENTAL

Antimicrobial susceptibility

Salmonella enterica strains isolated in the Bacteriology Laboratory between 2010 and 2012 were used in our study. *In vitro* antibiotic susceptibilities of 117 *S. enterica* isolates were examined using modified Kirby-Bauer disc diffusion assays in accordance with Clinical and Laboratory Standards Institute (CLSI).[7] Standard ampicillin (10 µg), cefotaxime (30 µg), ciprofloxacin (5 µg) sulfamethoxazole/trimethoprim (23, 75/1.25 µg) (Oxoid, United Kingdom) discs were used to detect resistance. The other part of the study was performed in ampicillin- resistant strains. Minimum inhibitory concentration (MIC) values of the isolates were also determined using broth microdilutions in accordance with the recommendations of the CLSI and *S. enterica* ATCC 04059 was used as a control strain.[8]

DNA isolation

DNA isolation was performed for use in polymerase chain reaction (PCR) studies. For this purpose, isolates were suspended and homogenized in 200 µL of sterile ultrapure water. The isolates were then incubated in a heat block at 95°C for 10 min. Microtubes were centrifuged at 13,000 rpm for 5 min. The supernatants were transferred to sterile microtubes and stored at -20°C for use in PCR studies.

Serotypes and epidemiologic relation

The strains were serotyped by the Turkish Public Health Agency, National Microbiology Reference Laboratory. The epidemiologic relations of the isolates were analyzed using PCR with enterobacterial repetitive intergenic consensus (ERIC)-2 and ERIC-1R primers.[9] To evaluate similarity between these isolates, Jaccard coefficients were derived from the banding patterns. Dendrograms were constructed according to the unweighted pair group with arithmetic mean method, using Jaccard coefficients and MEGA software, version 4.0.

Beta-lactamase genes

bla_{CTX-M}, bla_{TEM}, bla_{SHV}, bla_{PER} genes were determined using primers targeting the relevant regions[10-12] using the conventional multiplex PCR method. PCR assays were run in 25 µL amplification mixtures composed of 5 µL bacterial DNA template, 2.5 µL Taq buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 30 pmol forward and reverse primers, and 1.25 U Taq polymerase.

Virulence genes

Six different virulence genes were analyzed in two different multiplex PCR reactions, using primers targeting the relevant genes.[13,14] For this purpose, a 5-µL bacterial DNA template, 2.5 µL Taq buffer, 1.5 mM $MgCl_2$, 200 µM dNTP, 20 pmol forward and reverse primers, 1.25 U Taq polymerase were prepared in 25 µL volume.

RESULTS

As a result of the disc diffusion test, ten (8.5%) out of 117 *S. enterica* isolates were detected as resistant to ampicillin. These resistant strains were susceptible to ciprofloxacin, cefotaxime,

sulfamethoxazole/trimethoprim. The ampicillin MIC range of the isolates was found as 512-128 µg/mL. The strains were divided into seven different clusters based on ERIC-PCR results. The detected serotypes were as follows; S. Enteritidis (n=5), S. Infantis (n=2), S. Typhimurium (n=1), and S. Corvallis (n=1). One isolate could not be serotyped. Five strains involved bla_{TEM} genes, two strains contained bla_{CTX-M} genes. bla_{PER} and bla_{SHV} genes were not encountered. *InvA*, *pipD*, *sopB*, which are virulence genes, were detected in all strains, *sifA* in seven, *pefA* in four, and *sopE* in three strains.

DISCUSSION

The *Salmonella* genus comprises many members. Some *Salmonella* serotypes are known to be more commonly isolated. In light of this information and existing data; the most frequently isolated *Salmonella* serotype is known to be S. Enteritidis.[15] In our study, five of the ampicillin resistant *Salmonella* strains were determined as S. Enteritidis. In 2014, Maraki and Papadakis[4] determined S. Enteritidis as the most commonly isolated serotype of *Salmonella* (37.3%). In another study by Ozdemir and Acar,[16] S. Enteritidis was determined as the most commonly isolated serotype from *Salmonella* isolates collected from 4 different provinces of Turkey. Our results show consistency with the literature.

Serotyping is the basic phenotypic method for epidemiologic investigation of isolates. Nonetheless, it cannot differentiate strains of the same serotype. Genotypic methods such as pulsed-field gel electrophoresis (PFGE), ERIC-PCR, and repetitive element palindromic PCR can distinguish the strains more effectively. Although PFGE is the gold standard method for fingerprinting, due to the lack of equipment and to avoid protocols lasting four-five days, a simpler method of ERIC-PCR was preferred.[17,18] In our study, strains were divided into 7 unrelated clusters using ERIC-PCR with an acceptable (≥0.90) discriminatory index value of 0.92. When the literature and data obtained in this study are evaluated, ERIC-PCR is considered to be a useful and easily applicable method for genotyping of strains.

Recently, *Salmonella* strains have shown resistance against many antibiotic groups. Ampicillin, which is a member of the beta-lactam antibiotics, is the first-line agent used in the treatment of *Salmonella* infections. *Salmonella* strains that are resistant to ampicillin and other beta-lactams pose a risk for public health.[5] According to our disc diffusion results, ampicillin-resistant strains are susceptible to other groups of antibiotics such as ciprofloxacin, cefotaxime, and sulfamethoxazole/trimethoprim.

According to data gained abroad, the rate of ampicillin resistance against *Salmonella* varies from one country to another. As for studies abroad; in India, ampicillin resistance in *S. enterica* isolates was detected as 25% in 2011.[19] It was 33% in *Salmonella* isolated from children in Cambodia,[20] 46% in Korea,[21] 55% in Spain,[22] and 8% in the United States.[23] In Turkey, there have been a few studies of clinical *Salmonella* strains that were isolated from children's hospital. The rate of ampicillin resistance in *Salmonella* strains were determined as 25.8% in 2012,[24] and 19% in 2014.[25] However, resistance rates were higher than in our study. According to the our knowledge, the stress of starting antibiotic treatment empirically in pediatric patients, before culture results, might be responsible for higher ampicillin resistance rates than in our study.

In our study, five isolates with bla_{TEM} genes and two isolates with bla_{CTX-M} genes were found. bla_{CTX-M}-positive isolates were in the S. Ifantum serotype. Four of five bla_{TEM} gene positive isolates were in S. Enteritidis serotype, and the remaining strain was in S. Typhimurium. Although the most common beta-lactamase genes of *Salmonella* isolates are variants of bla_{CTX-M} and bla_{SHV},[26] no bla_{SHV} gene was found in isolates used in our study. Different rates of beta-lactam resistance genes were reported in studies conducted abroad. Among 20 ampicillin-resistant *Salmonella* isolates, beta-lactamase genes were found to be bla_{SHV} 100%, bla_{TEM} 85%, bla_{CTX-M} 5% in one study;[2] and in 90 beta-lactam–resistant strains in Spain it was bla_{TEM} 22%, and bla_{CTX-M} 1%.[22] In the Netherlands, a study with 34 *Salmonella* strains isolated from humans and the environment, Hasman et al.[6] detected the bla_{TEM} gene in 19 (55%) strains, the bla_{CTX-M} gene in six (17%), and the bla_{SHV} gene in three (8%) strains; all 34 isolates were found to be resistant to penicillin. A study conducted on S. Typhimurium in 2011 in Turkey detected 23% bla_{CTX-M} gene, 76% bla_{TEM} gene, and 100% bla_{SHV}. The bla_{PER} gene could not be detected in any isolates[27].

Salmonella bacteria carry many different and complex virulence factors. Investigating the presence of virulence factors coded as different pathogenicity islands will guide us in the matter of discovering *Salmonella* pathogenesis. Our study is thought to be the first in Turkey to investigate the virulence factors of *Salmonella*, at least three virulence factors were detected in all strains. The *invA* gene was determined in all isolates involved in this study, and it was present independent from conditions such as serotype and resistance genes. Dione et al.[26] detected the *invA* gene in 99.5% of strains in a study. Another study conducted by Smith et al.[5] in 2010 encountered the *invA* gene in all. The determination of high levels of the *invA* gene in different regions despite different serotypes and antimicrobial susceptibility profiles indicated the existence of a preserved region in this gene. Thus, the idea of using this gene for rapid diagnosis of *Salmonella* with PCR as a target region has arisen, and this idea has led to studies with positive outcomes.[28]

Generally, the *sopE* gene, which has been shown to have the lowest prevalence, has been associated with epidemic cases.[26,29] In our study, the presence of three strains that involve this gene in the same group according to ERIC-PCR and their isolation in a short period of time indicated that they may have been isolated after a community- onset epidemic.

Hughes et al.[14] detected *pipD* and *sopB* genes in all strains and these virulence factors have been associated with enteritis. The detection of these genes in all resistant isolates indicated a possible relationship between these virulence factors and resistance. A study conducted by Dione et al.[26] and our data gave similar results. Also, Khoo et al.[30] detected alterations

in beta-lactam resistance as a result a mutations created in these two genes. Although our study did not directly show the relationship between resistance and virulence factors, it showed the necessity for further extensive studies about the relationship between these two factors.

The *pefA* gene, which was detected in four strains at the present study, may be located on the same or different plasmid with different virulence (*spv*) genes. Only a fraction of *Salmonella* serotypes carries different-sized plasmids, which are known as serovar specific. However, it is known that not every plasmid-carrying serotype includes the *pefA* gene. Therefore, it is thought that the *pefA* gene has a lower prevalence compared with other virulence factors.[31] Hughes et al.[14] showed that only three isolates out of 32 involved the *pefA* gene. Skyberg et al.[13] performed a study on *Salmonella* of different serotypes, the *pefA* gene was found in 11 of 152 strains, and it was only present in serotypes of S. Typhimurium and S. Enteritidis. The low prevalence of the *pefA* gene in our study is compatible with the results of the other studies. Additionally, three out of four *pefA* gene-carrying strains belonged to the S. Enteritidis serotype, and this result is also consistent with the literature data.

The *sifA* gene, which enables the sustained vitality of *Salmonella* bacteria in macrophages, was detected in seven strains in our study. Hur et al.[32] detected the *sifA* gene in all of 42 strains. Skyberg et al.[13] determined the *sifA* gene in 137 out 158 isolates. The existence rate of the *sifA* gene in this study was similar to that of studies conducted abroad.

CONCLUSION

As a result, in light of studies both in our country and abroad, it is known that there are *Salmonella* strains that are resistant to antimicrobial agents, some of which are beta lactamase-producing. Further investigation on the resistance and virulence profiles of *Salmonella* strains will enable us to better understand the pathogenesis of infections and to be able to take better measurements and give proper treatments.

ACKNOWLEDGEMENTS

We are grateful to the UEPLA team for the serotyping of the strains as a surveillance program of the Public Health Institution of Turkey.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):212-218
DOI: 10.4274/tjps.55376

ORIGINAL ARTICLE

Opinions of Community Pharmacists About Collaboration with General Practitioners: A Descriptive Pilot Study After New Legislation in Turkey

Serbest Eczacıların Aile Hekimleri ile Çalışmaları Konusundaki Düşünceleri: Türkiye'deki Yeni Yasal Düzenleme Sonrası Tanımlayıcı Bir Pilot Çalışma

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ABSTRACT

Objectives: Committed legal regulations in primary healthcare services have resulted in certain issues and requirements about community pharmacy practices in Turkey. A professional collaboration between general practitioners and pharmacists is essential for the continuity of care in primary care settings. Therefore, the aim of this was study to explore community pharmacists' opinions on collaborative care along with new primary care regulations.

Materials and Methods: A web-based questionnaire was implemented during a period of five months to a voluntary sample of community pharmacists. The questionnaire consisted of multiple-choice questions that allowed participants to select more than one answer wherever appropriate.

Results: One hundred ninety-seven community pharmacists responded to the questionnaire; 66% were younger than 39 years and had less than 15 years' experience in the profession. In general, the pharmacists are willing to be involved in maintaining continuity of care, especially in patient counselling. However, 10.7% of participants indicated that the main reasons for not being actively involved in patient care were 'mainly dealing with explaining prescription co-payment procedures' and 'having unfavorable attitudes of other healthcare professionals'. They believed that their contributions were restricted by laws and regulations (14.2%) as well as declined perceived reputation (53.8%) of the profession among the public.

Conclusion: Revealing misperceptions among patients and healthcare professionals, overcoming limitations of current practice and an understanding of each profession's responsibilities may lead to formation of a new and more effective model of care.

Keywords: Patient-centered practice, interprofessional, collaboration, pharmacist, general practitioner

ÖZ

Amaç: Birinci basamak sağlık hizmetlerinde yapılan yasal düzenlemeler, serbest eczacılık hizmetlerinde bazı hususların ve ihtiyaçların ortaya çıkmasına neden olmuştur. Birinci basamak sağlık hizmetlerinde eczacılar ve aile hekimleri arasındaki profesyonel işbirliği, sağlık bakım hizmetinin devamlılığının sağlanması için gereklidir. Bu nedenle bu çalışma, serbest eczacıların birinci basamak sağlık hizmetlerindeki yeni düzenlemeler ile, işbirliği şeklindeki bakım hizmetleri hakkındaki görüşlerini ortaya koymayı amaçlamaktadır.

Gereç ve Yöntemler: İnternet sayfası tabanlı anket çalışması, 5 ay süre ile, gönüllü olarak katılım sağlayan bir serbest eczacı örneklemine uygulanmıştır. Anket, çoktan seçmeli sorulardan oluşmaktadır ve gerektiğinde katılımcıların birden fazla seçenek seçmesine olanak sağlamaktadır.

Bulgular: Çalışmada, 197 serbest eczacı soruları cevaplamıştır; katılımcıların %66'sı >39 yaş ve 15 yıldan daha az tecrübesi olan eczacılardır. Genel olarak, eczacılar bakımın devamlılığını sağlamak konusunda, özellikle hasta danışmanlığında, katılımcı olmaya istekli olduklarını belirtmiştir. Ancak, katılımcıların %10.7'si, aktif olarak hasta bakımına katılamamanın temel nedeninin; 'reçete geri-ödeme prosedürleri ile uğraşmak' ve 'diğer sağlık çalışanları tarafından teşvik edici olmayan tavırlar' olduğunu belirtmiştir. Katılımcılar, bu katkılarının var olan yasal düzenlemeler (%14.2) ve toplumda azalan saygınlık (%53.8) nedeniyle kısıtlandığına inanmaktadır.

Sonuç: Hastalar ve sağlık çalışanları arasında var olan yanlış algıların açığa çıkarılması, var olan hizmetteki sınırlamaları ortadan kaldıracak ve her mesleğin sorumluluklarının anlaşılması, yeni ve daha etkin bir bakım modelinin oluşturulmasına yardımcı olacaktır.

Anahtar kelimeler: Hasta-odaklı uygulama, mesleklerarası, işbirliği, eczacı, aile hekimi

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Received: 14.02.2017, Accepted: 17.08.2017

INTRODUCTION

An improvement in the healthcare system depends on stakeholders who perform the required responsibilities and expanded professional competencies and skills. In terms of chronic disease management, both primary and secondary care health professionals should play active roles in patient care.

In Turkey, the National Health Service in primary care has been revised and general practitioners' (GPs) roles have been expanded since 2010. The new legislation on the primary health care process was introduced in 2013 and revised in 2015. By January 2013, electronic prescribing also began to be implemented in Turkey. Previously, GPs were responsible for monitoring chronic diseases as well as providing preventive health services along with the nurses in primary care settings. Before the new legislation, GPs practicing in primary care settings were only allocated in a small and a limited number of community clinics in a city where they only have close contact with nurses for the patient care process. Patients were free to attend any community clinics for their monitoring and prescriptions according to their preferences. After the legislation, the patients are obliged to attend a certain healthcare center and monitored by an assigned GP according to their home addresses, and were not allowed to change their doctor within the first 3 months. Patients are guided to visit GPs first before attending secondary or tertiary care settings where health expenses could not be covered by the national health insurance.

Groups of GPs (e.g. minimum 2 doctors) are also allocated in certain healthcare centers according to the population size and number of patients to be served. They are entitled to serve a minimum of 3500-4000 patients, work a total of 40 hours per week depending on the total population number in a district. If the assigned number of patients for GPs declines below 1000 patients in a minimum 2 consecutive months, their agreements with the national health service becomes invalid. With the implementation of new legislation, the roles are merged between GPs and nurses, where monitoring of chronic diseases is focused by doctors and preventive health services are undertaken by nurses, which has led to disunity of health services in primary care. There is also a growing interest in clinical pharmacy practice about expanding the role of pharmacists to be actively involved in the patient care process. Unlike most European countries, clinical pharmacy is a new and emerging concept in Turkey and has gained the attention of pharmacists, as well as other healthcare professionals.

According to a report of the Turkish Ministry of Health, patients' primary care visits increased to 2.7 visits per patient/year in 2010 compared with 0.9 visits per patient/year in 2002.[1] A recent study conducted by Çiceklioğlu et al.[2] indicated that GPs' professional identities have shifted to businesspeople from healers, workloads are increased, and more patients behave as demanding consumers as result of the Turkish Health Transition Program. Given the fact that there are a large number of community pharmacists, a rational use of pharmacy services is inevitable. Therefore, novelties in primary care services create opportunities for both professions in terms of shared responsibilities in the patient care process.

The role and contribution of pharmacists in patient monitoring has been well documented in the literature, mainly in chronic disease management;[3-5] however, there was a low tendency to report the perceptions, attitudes, and opinions of healthcare providers. Wüstmann et al.[6] determined the views and attitudes of pharmacists and GPs in Germany towards each other regarding collaboration. They concluded that although the frequency of contact was low, there was a good level of trust between both professions. Moreover, pharmacists and GPs regard that their responsibilities are important in maintaining patient adherence to drug treatment in the long-term, but more frequent co-operation is necessary.

Bryant et al.[7] explored the GPs' perceptions on the expanding role of the community pharmacist in clinical medication reviews. They showed that GPs regarded pharmacist's skills as valuable, supportive in repeat dispensing schemes, and helpful in patients' medication management; however, they indicated that the value of services provided by a pharmacist should be balanced between improved patient outcomes and resources (time and funding) required.

On the other hand, it has been shown that[8] more than half of all GPs support the involvement of pharmacists in the activities of providing public health education, contacting GPs on matters related to prescribing and prescription errors, and referring patients who exhibit drug-related problems. However, less than half have doubts regarding pharmacists' role in a smoking cessation program and the provision of drug information to doctors.

According to Van et al.[9], attitudes towards collaboration are strongly related to communication, mutual respect, willingness to work together, and the recognition of roles, which are influenced by trust and expectation. One of the common barriers indicated by Hughes and McCann[10] was the professional image of community pharmacists, which also affects public perception and awareness with regards to the care they receive from pharmacists. Furthermore, the integration of pharmacists and remuneration are also considered as barriers.[11]

The factors that affect pharmacist-GP collaboration have also been identified by Rubio-Valera et al.,[12] who showed the associated factors as perception of usefulness, the clinic manager's interest, professional attitude, geography, and legislation. Similar findings were documented by Saramunee et al.,[13] indicating that community pharmacies were a good source for advice on medicines and minor ailments but were less supportive in public health services. The main barriers that can interfere with service use were listed as the perceptions of both the general public and other health providers towards pharmacists' competencies, privacy and confidentiality in pharmacies, high dispensing workload, and inadequate financial

support. Therefore, a clearer understanding of the perceptions of health professionals is crucial for establishing a continuous collaboration in the patient care process.

Bryant et al.[14] indicated that there were significant barriers preventing community pharmacists from becoming involved in clinical services because of the lack of acceptance of clinical roles in disease management. However, GPs strongly agreed with the pharmacists' role in patient counselling, education, compliance, and reporting adverse events.

Many studies have reported GPs' perspectives and opinions on a collaborative care; however, few studies have searched for pharmacists' opinions. It is important to reveal the level of willingness of participation and professional trust in the competencies of pharmacists in collaboration. Furthermore, no studies have been conducted in Turkey among health professionals in order to identify their opinions and perceptions on a shared care process after the introduction of the new health transition program. Therefore, the aim of this study was to identify community pharmacists' opinions on a shared care process and their willingness to be involved in patient monitoring in order to improve quality in primary health care settings.

MATERIALS AND METHODS

The survey study was undertaken via a web-based questionnaire (www.teb.org.tr) with the support of the Turkish Pharmacists Association (TEB). The questionnaire was initially designed by the researchers in view of the literature, and sent by e-mail to 20 pharmacists from different cities in order to clarify the meaning and understanding of questions. According to the responses and comments from pharmacists, some questions were re-worded and some others were omitted from the study. The revised final questionnaire comprised a total of 27 questions; nine regarding the pharmacist's/pharmacy's demographics, and other questions were related to the pharmacist's opinions on the implementation of the new legislation and collaboration with GPs. The participants were allowed to choose more than one answer wherever appropriate.

No similar studies have been undertaken among the Turkish pharmacists previously; therefore, the researchers had no reference values to calculate a sample size based on any assumptions for this study. Community pharmacists who are willing to participate in the study entered the web system using either their social security institution identification number or a prescription issue entry number, then completed the questionnaire on the web page of the TEB.

Ethics committee approval was not sought for this study and it was undertaken collaboratively with the TEB. The association is an official representative council of pharmacists in Turkey, and the survey was an initiative of the association in order to explore the perspectives of its members about the newly-emerged situation in the healthcare system in order to improve pharmacy services to be re-established in Turkey.

Statistical analysis

At the end of the data collection period, the responses were collected through a web-based program and the data were analyzed by statisticians using SPSS-11.

RESULTS

During five-month period, a total of 200 pharmacists responded to the questionnaire; three questionnaires were excluded from the analysis due to a lack of data. Two thirds of the pharmacists who participated in this study were aged younger than 40 years and had less than 15 years' experience in the profession. The majority of pharmacies were located near a GP practice and dispensed 26-50 prescriptions per day. The details of the participants are summarized in Table 1.

Table 1. Characteristics of the participants in the study

	n (%)
Sex	
Male	118 (59.9)
Female	79 (40.1)
Age	
≤30	53 (26.9)
31-39	77 (39.1)
40-49	41 (20.8)
≥50	26 (13.2)
Year of experience in the profession;	
≤5 years	42 (21.3)
6-15 years	89 (45.2)
16-25 years	39 (19.8)
≥26 years	27 (13.7)
Educational level;	
University	168 (85.3)
MSc/PhD (postgraduate qualification)	29 (14.7)
Location of pharmacy;	
Main street	48 (24.4)
Near hospital/medical clinic	35 (17.8)
Near GP practice	92 (46.7)
City center/in the mall	22 (11.2)
Number of prescriptions dispensed per day	
1-25	34 (17.3)
26-50	89 (45.2)
51-75	49 (24.9)
76-100	19 (9.6)
101-125	4 (2.0)
126-150	1 (0.5)
≥151	1 (0.5)
Number of patients' visit to the pharmacy per day	
1-25	19 (9.6)
26-50	55 (27.9)
51-75	49 (24.9)
76-100	45 (22.8)
101-125	17 (8.6)
126-150	8 (4.1)
≥151	4 (2.0)

GP: General practitioner

Influence of new legislation on pharmacy practice

According to the study results, the change in healthcare legislation in Turkey has caused 19.8% of pharmacists to change the location of their pharmacies in order to be near a GP practice. Pharmacy health services were also affected by this new legislation and its impact on pharmacies was in both extents. More than 45% of the pharmacists agreed that the number of drug varieties (46.2%), dispensed drugs that should be prescribed by a specialist (47.2%), and patients' visits to pharmacies for counselling (48.7%) had increased, whereas the number of dispensed prescriptions (41.6%) had decreased.

Professional interactions with GPs

In this study, most of the pharmacists reported the frequency of their contact with GPs about patients' prescriptions and health status was 1-5 times per week (43%) or never (19%). Instead, the majority of GPs had never (52%) or 1-5 times per week (36%) contacted pharmacists. The pharmacists who contacted GPs 1-5 times per week mainly dispensed 26-50 prescriptions per day (50.5%) and were located near the GP practice (48.2%), a hospital/health center (22.3%), on a main street (22.3%) or in a city center/near a shopping mall (7.2%). Interestingly, the pharmacists who never contacted GPs were also located near the GP practice (51.3%). There was no difference between pharmacists who did and did not contact GPs in terms of sex, age, the number of dispensed prescriptions, and the location of the pharmacy ($p<0.05$). Only 16.2% of the pharmacists stated having a positive communication with GPs (Table 2).

With regards to the common issue of communication between a pharmacist and GP, GPs mainly contacted pharmacists regarding the reimbursement status of a drug from National Social Security (55.8%), whether a patient had sufficient medication until the next visit (13.7%) or eligibility of a drug in the market (11.7%). Similarly, pharmacists contacted GPs if any inconsistency was recognized between a prescription and a patient's health report (74%) or for correcting any errors in the prescriptions (16.2%). Unfortunately, neither GPs nor pharmacists contacted each other regarding drug use, dose and dosing errors or any potential drug interactions, according to the pharmacists' opinion.

In consideration of the pharmacist-GP relationship, although over half of the pharmacists indicated that their professional relationship with GPs had not changed by this new legislation, the proportion of pharmacists who believed that communication regarding a patient referral to a GP and a GP's referral to a pharmacy for counselling (31% vs. 24.4%) and the provision of drug information by a pharmacist to GPs (28.4%) were increased.

Table 2. Pharmacists' professional communication with general practitioners

Behaviors of the GPs perceived by pharmacist	n (%)
Distant relations and asking questions to GPs not allowed	69 (35.0)
Closed to any kind of communication	68 (34.5)
Descriptive, informative and constructive	32 (16.2)

GPs: General practitioners

Perceived roles of pharmacists in collaborative care

The participants considered the role of a pharmacist in drug treatment as counselling/providing drug information (47.2%), joint decision-maker with doctors (28.4%), monitoring drug use (17.8%), a decision-maker (3.6%), and a pharmacoeconomic supervisor in drug use (3.0%). The participants who considered pharmacists as a consultant (93 out of 197) and a joint decision-maker (56 out of 197) were mainly aged 31-39 years (38 out of 93 vs. 19 out of 56) and had 6-15 years of experience (40 out of 93 vs. 25 out of 56). The pharmacists who were aged over 50 years (26 out of 197) considered the pharmacist's role more likely to be counselling/providing drug information (17 out of 26).

Eighty-four percent of pharmacists (n=165, of whom 42% were female and 58% were male; 38% were 31-39 years and 29% were <30 years) believed that the role of pharmacists in counselling should focus on providing information about drugs and patients' diseases. Nevertheless, the provision of information by pharmacists about drug interactions, the selection of an appropriate drug, dose forms and dosage were not acknowledged by the participants.

With respect to the influence of new legislation on the patient–pharmacist relationship, more than half the pharmacists reported that communication with patients had increased when it came to providing information about drugs and reimbursement status. Although 50.8% of the participants indicated that the perception of pharmacists' roles in preventive public health services (smoking cessation, vaccines, healthy nutrition) were not changed, an increased perception was also reported by 37.6% of the responders.

Preferred roles of pharmacists in collaborative care

The participants (%) believed that if they had access to detailed patient medical records through the National Social Security System they could;

- Counsel about public and preventive health (family planning, vaccines, obesity and smoking cessation) (65.5%),

- Provide drug information to doctors about appropriate drug and dose forms as well as drug interactions (60.9%),

- Advice drugs for a minor illness (such as cold and flu, acne, fungal infections and scabies) (79.2%) to patients,

- Maintain patient care in collaboration with doctors according to the treatment guidelines (58.9%),

- Review patient's drugs and laboratory results and refer to a doctor early if required (65%),

- Work on pharmacovigilance (75.6%),

- Help to increase patient adherence (70.1%),

- Dispense repeat prescriptions without the need for a patient to be seen by a doctor (61.9%).

Table 3. The pharmacists' reasons for not being able to be involved in a patient's drug treatment

Reasons	n (%)
Too much time to explain the prescription co-payment	21 (10.7)
Lack of patients' time	4 (2.0)
Slow data entrance system of the National Social Security System	7 (3.6)
No reimbursement for such specific service	2 (1.0)
Unfavorable attitudes of other health care professionals	21 (10.7)
Unfavorable attitudes of patients	8 (4.1)
Legal limitations	28 (14.2)
Perceived status of the pharmacy profession among the public	106 (53.8)

The participants were asked to give their opinions on their active roles in a patient's drug treatment, including over-the-counter drugs. Approximately 76% of the participants (61 females, 88 males) believed they had active roles in drug treatment, of which 46% generally worked near a GP's practice and received and/or dispensed 26-50 prescriptions per day. However, a majority of the pharmacists (54%) stated not being involved in collaborative drug treatment processes in daily practice (Table 3).

Moreover, the participants highlighted professional needs in order to provide counselling services to patients and healthcare professionals. Forty-two percent of participants indicated that they should be acquainted with the legal regulations on drug reimbursement, whereas 37% believed that they should have a continuous/uninterrupted transfer of electronic patient records from GPs. Only 3% reported that they needed to follow professional educational programs in order to provide such services.

Reimbursement of pharmacy services

In regards to reimbursement of such services provided by pharmacies, the participants indicated that reimbursement could be gradually incremented on the basis of the number of prescriptions and/or drug boxes (34.5%), the number of health services provided and documented by a pharmacist (20.3%), or a fixed amount according to the number of working hours per month (18.3%).

DISCUSSION

Along with the initiation of new legislation, there would be an opportunity for close collaboration between pharmacists and GPs in the patient monitoring process and other primary healthcare activities.

Previous studies have already focused on potential barriers for communication among healthcare professionals and collaborative healthcare services in different practice settings, such as medication review, medicine management, patient/health education,[15] and prescribing/prescription errors and early referrals to GPs.[8,11,16]

A study from Malaysia has shown similar findings, where a majority of community pharmacists were still focusing on counselling for nutritional supplement, cough and cold, and diabetes, and providing screening tests for blood pressure and blood sugar levels. Generally, GPs were supportive towards the involvement of pharmacists in extended pharmacy services but they were uncertain about the knowledge and skills. Most of the GPs indicated that they would like to collaborate with community pharmacists on improving patient therapeutic outcomes (~55%) and welcome patient referral from community pharmacies for further medical evaluation (~70%). However identified barriers was listed as lack of time, shortage of man power, lack of collaboration between healthcare professionals, legal and regulatory constraints, and lack of self-efficacy.[17]

According to the results of this study, communication between pharmacists and GPs was infrequent and described as distant and did not allow for further discussion; furthermore, collaboration in decision-making or shared information during patient care did not seem to be feasible in routine practice. Information shared by health professionals was mainly on technical or bureaucratic issues rather than individual professional knowledge. Therefore, the perception of each profession and their expectations were restricted; it would take time to establish the inter-professional trust between GPs and pharmacists in taking responsibilities of patient care.

In the present study, it was also reported that community pharmacists had conflicting opinions on working in collaboration with GPs in primary care. Although there has been apparent enthusiasm about being involved in patient care, pharmacists were not in favor of providing such services with no reimbursement. Moreover, 10.7% of participants indicated that they were exhausted about explaining prescription co-payment procedures and unfavorable attitudes of other healthcare professionals, which were the main reasons for not being actively involved in drug treatment.

Community pharmacists were enthusiastic about providing drug information; however, they were reluctant to take an active role in patient monitoring because of legal limitations, reimbursement problems, and the perceived reputation of community pharmacists among the public.

With the implementation of new legislation, 19.8% of the pharmacists had to change the location of their pharmacies. Through a dissemination of increased numbers of GP practices around different locations, pharmacies also had to change their location in order to maintain their income and sustain the quality of pharmacy services. It is necessary to indicate that all community pharmacists are self-employed in Turkey. Therefore, macro- or micro- economic changes may affect pharmacies' income due to relatively high leases and low fees for professional counselling.

Study limitations

The main limitation of the study is the number of participants. The questionnaires were not delivered to pharmacists personally, instead they was uploaded on the website on the 'news' section of the TEB web page. Pharmacists generally visit the website in order to reach updated information about legal procedures in pharmacy practice or reimbursement of particular drugs, not to complete questionnaires. Therefore, not many pharmacists were likely to participate in the study and the web-based questionnaire was not the most appropriate method for this group of pharmacists.

The study was undertaken soon after the new legislation was implemented; therefore, the participants might not have clear/robust views about its impact. It would be better to surrogate the questions in the future when the implication of the legislation will be more sustained. Although the results of this study might not reflect the opinions of all pharmacists, it can still be considered as a critical overview of pharmacy practice in primary care.

It would also be interesting and invaluable to reflect and compare a sample of GPs' views and expectations along with the participated pharmacists at the same time, which would create a perspective for future studies. However, it could not be manageable for this study; therefore, it can be considered as another limitation.

It is believed that the study results managed to highlight some of the main problems and eventual solutions for a shared care process in primary care. By the results of this study, another pilot project was initiated by the TEB in order to identify pharmacist's role in monitoring patients with hypertension in collaboration with GPs. The professional bodies found an opportunity to lead the legal authorities and guide the pharmacists in terms of professional improvements in the country.

CONCLUSION

Such practice implications are unique for pharmacists where the concept of clinical pharmacy practice is not well-established in countries such as Turkey. Therefore, determination of needs and expectations, and identification of barriers and challenges in local/national health care settings will expand the ideas of practitioners in improving collaborative care.

Local implementation of European healthcare models for the continuity of care in primary care settings may enlighten different countries in order to enhance health services; however, variations in regulations of health systems may lead to occurrence of reluctance in contribution. An integration of clinical governance within a primary care process by active involvement of pharmacists may yield shared-decision making in drug therapy, reduce workloads of GPs, and also enhance patient satisfaction.

Community pharmacists are willing to participate in the patient care process and they are best-positioned to provide drug information and monitor patients' health outcomes in pharmacies. A delegation of responsibilities by laws and regulations would give confidence to pharmacists and maintain collaborative care for patients. A communication pathway between GPs and pharmacists should be re-arranged by a healthcare model that is designed according to technical issues on prescriptions, which meets the mutual professional expectations of each profession.

ACKNOWLEDGEMENTS

We would like to thank the Turkish Pharmacists Association for their kind support and Mr. Ömer Kutlu and Ms. Özge Bal for their contribution during data analysis and Mr. İlker Mintaş (M.D, General Practitioner) for his invaluable advices.

Conflict of Interest: No conflict of interest was declared by the authors.

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Turk J Pharm Sci 2018;15(2):219-230
DOI: 10.4274/tjps.63497

REVIEW

Carbon Quantum Dots: Synthesis, Characterization and Biomedical Applications

Karbon Kuantum Noktaları: Sentez, Karakterizasyon ve Biyomedikal Uygulamalar

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ABSTRACT

CQD are small carbon nanoparticles smallerl than 10 nm comprising distinctive properties, which have become an obligatory tool for traceable targeted delivery, biomedical research, and different therapy applications. The objective of the present work was to consolidate the current literature on the synthesis, characterization techniques, and biomedical applications of CQD. Two types of synthetic methods viz. top-down approach and bottom-up approach were used for the synthesis of CQD. The top-down approach includes the arc-discharge method, laser ablation method, and electrochemical method. The bottom-up approach includes the thermal method, microwave-assisted method, hydrothermal and aqueous method, and the template method. In this review, we explain the recent progress of CQD in the biomedical field, focusing on their synthetic methods and characterization, followed by different applications. Carbon dots have extensive adequacy for *in vivo* and *in vitro* bioimaging and drug delivery studies. Although more cytotoxicity studies of carbon dots are needed, the data above suggest a bright future for carbon dots in drug delivery and bioimaging studies.

Key words: Carbon quantum dots, nanoparticles, quantum yield, carbon dots, photoluminescence, nanocomposites

ÖZ

KKN, ayırt edici özellik olarak, 10 nm'den daha küçük boyutu olan, küçük karbon nanopartiküllerdir ve izlenebilir hedeflenmiş salım, biyomedikal araştırma ve farklı terapi uygulamaları için zorunlu bir araç haline gelmiştir. Bu çalışmanın amacı, KKN'nın sentezi, karakterizasyon teknikleri ve biyomedikal uygulamaları ile ilgili güncel literatürü bir araya getirmekti. KKN sentezi için iki tip yapay yöntem yani yukarıdan aşağıya yaklaşım ve aşağıdan yukarıya yaklaşım kullanıldı. Yukarıdan aşağıya doğru yaklaşım, ark boşaltma yöntemini, lazer ablasyon yöntemini ve elektrokimyasal yöntemi içermektedir. Öte yandan aşağıdan yukarı yaklaşım, termal yöntem, mikrodalga destekli yöntem, hidrotermal ve sulu yöntem ve kalıp yöntemini içerir. Bu derlemede, CQD'nin biyomedikal alanda son dönemdeki ilerlemesini, sentetik yöntemlerine, karakterizasyonuna ve farklı uygulamalara odaklanarak açıkladık. Karbon noktaları, *in vivo* ve *in vitro* biyolojik görüntüleme ve etken madde salım çalışmaları için kapsamlı yeterliliğe sahiptir. Karbon noktaları için daha fazla sitotoksisite araştırması yapılmasına ihtiyaç duyulmasına rağmen, önceki veriler etken madde salımı ve biyolojik görüntüleme çalışmalarında karbon noktalarının parlak geleceğini göstermektedir.

Anahtar kelimeler: Karbon kuantum noktalar, nanopartiküller, kuantum verimi, karbon nokta, fotolüminesans, nanokompozitler

INTRODUCTION

Luminescent semiconductor nano crystals of size 1-10 nanometers with rich surface chemistry and unique optical properties are called quantum dots (QDs). Different compounds belonging to group 2 to 4 and 3 to 5, e.g., Ag, Cd, Zn, Hg, Se, Ln, Pb, P, and Te lead to the formation of QDs. These have become an obligatory tool for traceable targeted delivery, biomedical research, and different therapy applications. Long-term fluorescence imaging and the detection of the properties of these nanoparticles (NPs) have made them imperative in biomedical research. Different properties of QDs such as resistance to photobleaching, superior signal brightness, larger absorption coefficients, light emission, and contemporaneous excitation of different fluorescence colors make them unique, as well as indispensable. Advances in quantum surface chemistry studies have led to the development of polymer-encapsulated probes with high fluorescence properties that are stable under complex biologic conditions. To use QDs in biologic studies, it is extremely important to cap or passivate the ZnS or CdS layer around the QD (CdSe). This layering of ZnS or CdS leads to the improvement of the fluorescence quantum yield (QY) of QDs and provides protection against photo-oxidation. QDs have had a major impact in molecular diagnostics and in tissue molecular

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Received: 06.06.2017, Accepted: 10.08.2017

biology. The basic purpose in opting for QDs emerged from their incomparable and engrossing optical properties, which are not generally feasible for an individual molecule or bulk semiconductor, in addition to resistance against photobleaching. They have the ability to elucidate the pharmacokinetics and pharmacodynamics of drug applicant and serve as a "traceable" drug delivery system.[1-5] In 1984, the Russian physicist Ekimov first discovered QDs in glass crystals. After 1984, a systemic advancement in pharmaceutical sciences was driven, and a relationship was established between the size and band gap for semiconductor nano particles (by applying a particle in a sphere model) approximation to the wave function for bulk semiconductors. In the beginning, the studies were limited to CdSe/CdS and CdSe/ZnS QD, but later on, other "core-shell" QDs were developed and studied, e.g., ZnSe/CdSe.

Cadmium was the chief component in the composition of traditional QDs, but the use of cadmium was limited because leakage of cadmium ions leads to cytotoxicity.[5] The cytotoxicity caused by leaked cadmium ions led to the discovery of more biocompatible QDs. With the increasing demand for more biocompatible QDs, the emphasis shifted toward the development of cadmium-free QDs with high chemical stability, low toxicity, and different pharmaceutic applications. This led to the formation of different QDs such as carbon QD (CQDs), graphene QDs, and silicon QDs.[5,6] CQDs were first obtained through the purification process of single-walled carbon nanotubes through preparative electrophoresis in 2004. They were first indicated as 'carbon NPs (CNP)' but later adopted the name 'carbon dots (C-dots)', eliciting similar properties to inorganic QDs. C-dots have gathered wide attention and considerable potential in biologic applications. Also, biocompatibility has been touted as the main lead of C-dots in the branch of NPs applications. C-dots mainly consist of carbon, which is an abundant and nontoxic element, and they endow distinguished structural and electronic properties that are different from other NPs families.[5] Advanced device applications were achieved for QDs when their intrinsic properties were successfully tuned by doping with heteroatoms. Because of their biocompatibility, low toxicity, strong photoluminescence (PL), synthetic and photograph steadiness, C-dots have become a fascinating material for bioimaging and the detection of different analytes. C-dots ordinarily contain discrete, quasi-spherical NPs with sizes less than 10 nm. Sp_2- characterized CQDs consist of different functional groups such as carbonyl, ether, epoxy, amino, carboxylic acid, and a hydroxyl group on their surface. The presence of such groups on C-dots leads to their high hydrophilicity. The captivating PL properties of C-dots are subject to their edge shape, size, deformities, and surface passivation.[7] Highly bright CQDs, which are soluble in oil, can also be fabricated by hot injection with B and N co-doping by taking 1,2-hexadecanediol as a carbon precursor and surface passivation material.[8]

Advantages of carbon quantum dots

- Inexpensive-CQDs are inexpensive and abundant thus making them a rising star as a nanocarbon member.

- Photostability: stability and composition of CQDs lead to their greater photostability when compared with organic dyes and traditional QDs.

- Broader excitation and narrow emission: CQDs have a more sharply defined emission peak and broader excitation spectra than organic dyes and other cadmium-based QDs.

- Biologic properties: the superior biologic properties of C-dots, such as hydrophilicity, low toxicity, chemical stability, and good biocompatibility ensure their promised applications in biosensors, drug delivery, and bioimaging.

- Luminescence: greater luminescence as compared with other QD.

- Aqueous stability: CQDs have high aqueous stability as compared with other cadmium-based QDs and organic dyes.

- Electronic properties: outstanding electronic properties of carbon-based QDs as electron donors and acceptors cause the electrochemical luminescence and chemiluminescence, empowers them with broad potential in optronics, catalysis, and sensors.

- Chemical inertness: the chemical stability of C-dots is very high as compared with other QDs (traditional or metallic).[9]

Synthesis

Synthetic techniques for CQDs are categorized into two classes, 'bottom-up' and 'top-down' courses. These can be accomplished by means of chemical, electrochemical or physical systems.[10] Top-down strategies involve the fragmentation of carbon matter into CNPs, and strategies comprising arc discharge, laser ablation, and electrochemical approaches. Bottom-up strategies incorporate template strategy, thermal routes, pyrolytic process, hydrothermal and aqueous methods, supported synthetic technique, reverse micelle technique, microwave-assisted strategy, and substance oxidation.[11] The yield of CQDs could be enhanced during arrangement or post-treatment. Alteration of CQDs is additionally vital to obtain favorable surface properties, which are key for solvency and applications.[12]

- Moreover, carbon precursors, for example ground coffee, used tea leaves, grass, and light sediment are also used to develop C-dots. The development of C-dots by these precursors is plentiful and economical.[10] In addition, a green approach for easy and one-step synthesis of fluorescent C-dots can be achieved from wool (natural and nontoxic substance) for sensing the glycophosate detection.[13] Different synthetic approaches are mentioned in Figure 1.

Top-down approaches

Arc-discharge

This strategy can be employed to develop C-dots from crude carbon nanotube soot (sediment). The crude material (sediment) was oxidized with 3.3 M HNO_3 to introduce carboxyl groups, the resulted matter was then extracted with NaOH/basic solution of pH 8.4, a stable dark colored suspension was obtained. Gel electrophoresis was conducted to purify the extracted matter.[10] Separation of a quick-moving band of highly

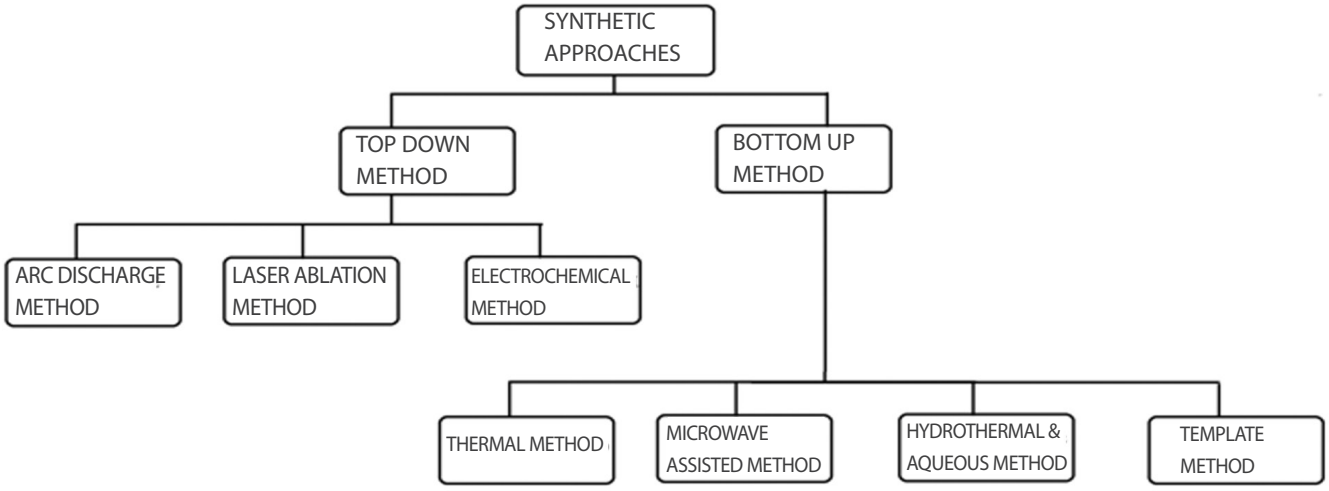

Figure 1. Different methods for the synthesis of carbon quantum dots

fluorescent carbon-dot was found to be 18 nm. Pristine and nitric acid oxidized carbon nanotubes were employed to outline the photoluminescent NPs; the latter being developed using the electric arc technique. Fluorescent NPs derived by the pristine carbon nanotube were hydrophobic and possessed limited distribution. On the other hand, the fluorescent NPs derived by the oxidized carbon nanotube had the capacity to accumulate when scattered in water because they were externally combined with oxygen and covered by a thin layer of carbon and demonstrated a more extensive division. CNPs obtained by the arc discharge method have low yield, in addition, the arc discharge soot method comprises a number of composite segments. However, the purification of these segments was hard.[11]

Laser ablation

In this method, synthesis of fluorescent C-dots is accomplished by laser irradiation of C-target (carbon target). Sun et al.[14] initially heated a blend of cement and graphite powder in order to prepare a carbon target, then fabricated CNPs by means of laser ablation/removal of the carbon target in a stream of argon gas conveying water vapor at 900 ˚C and 75 kPa. The nanoscale carbon particles were obtained in aggregated form possessing different sizes with no distinguishable PL. An aqueous solution of HNO_3 (nitric acid) was treated with the sample followed by refluxing for 12 h, later polyethylene glycol (PEG1500N) or poly propionyl ethyleneimine-co ethyleneimine was reacted with the sample, and the passivated C-dots were highly photoluminescent, having size of about 5 nm. With activation of these C-dots at 400 nm, the fluorescence QY was around 4% to more than 10%.[11] In addition, doping of C-dots with inorganic salts such as zinc acetate and Na_2S or NaOH further resulted in an enhanced QY, in which the dopants (e.g. ZnO and ZnS) possibly served as a helper passivating mediator for the C-dots. When activated at 450 nm, doped C-dots showed strong PL (QY 45%). PL C-dots of different colors were prepared in different solvents and aqueous medium, provided that the organic particles performed as passivation ligands.[10] Laser ablation has numerous points of interest; for example, effortlessness. Different types of nanostructures can be prepared through this technique, but this method requires a high amount of carbon matter for the development of carbon targets. Laser irradiation develops the different sized CNPs; large particles are effortlessly disposed of during the centrifugation process, so the resulting CNPs posses low yield, and the use proficiency of carbon matter was also less.[11] Carbon particles can also be fabricated in ethanol via the laser ablation technique.[15]

Electrochemical method

Lu et al.[16] used high-purity rods of graphite and profoundly situated pyrolytic graphite was used as an anode, with a partition of 2 cm, platinum wire as a counter electrode, followed by their installation into ionic fluid/water solution. The exfoliation of carbon matter was initiated by the application of static potentials. The process of exfoliation was conducted due to the complex exchange of anionic intercalation from the ionic fluid and anodic oxidative cleavage of water. Until the pH of exfoliation products was neutral, they were washed with ethanol and water. After separation by filtration and ultracentrifugation at 15,000 rpm at 20 ˚C, C-dots of 6-8 nm size were obtained with the QY of 2.8-5.2%. Yao et al.[17] settled an anode of spectrum-pure graphite ring and a cathode of a titanium tube at the middle of the electrolyzer. An insulated O-ring was used to separate the cathode and anode. Sterilized water was used for an electrolyte medium. Ultrasonic power and electrolytic voltage were applied at one time, and pure blue fluorescent C-dots of 2-3 nm size were quickly produced without any complex purification. The QY of the obtained C-dots was found as 8.9%. The resultant C-dots possessed a magnificent fluorescence effect and thermodynamic stability in aqueous solution.[11] The extent of C-dots can be managed by changing the current density. Larger C-dots with longer emission wavelength can be formed by lowering the current density.[10] Photoluminescent C-dots 3 nm in size are specifically

manufactured by electrochemical stunning of multi-walled carbon nanotubes.[2] Water-soluble C-dots can also be prepared by the chemical oxidation treatment of flour.[18]

Bottom-up approaches

Thermal routes

For the preparation of C-dots, the burning sediment of candles was used as a starting material. The treatment of sediment with an oxidant, for example, HNO_3 and H_2O_2/AcOH, resulted in the formation of C-dots. Polyacrylamide gel electrophoresis was conducted to isolate the obtained C-dots and demonstrated that C-dots with higher versatility possessed PL at shorter emission wavelengths. The QY estimations of the C-dots extended from 0.8% up to 1.9%. Sediment from natural gas was treated with HNO_3, followed by neutralization with $NaHCO_3$. Finally, purification was achieved through dialysis, which prompted the development of photoluminescent C-dots. Metal nanostructures were formed on the surface of C-dots by adding metal salts separately, including $AgNO_3$, $Cu(NO_3)_2$, and PdCl2, to the C-dots solution in the presence of a reducing agent (ascorbic acid).[10] The soot-based method is easy and clear.[19] However, the QY of the fluorescent CNPs is considerably less (<0.1%) and has no valuable purpose. An enhanced soot-based technique has been developed for the production of fluorescent CNP of 2-6 nm size and QY around 3%. There are 3 discrete improvements in the accompanying adjusted method. Initially, a basic isolation method for a small-sized fluorescent molecule from heterogeneous particle mixture is developed. The technique is appropriate for the synthesis of these particles (on milligram scale). The second method demonstrates that large particles are less fluorescent than smaller ones and hence isolation of small molecules enhances the QY (<0.1% to ~3%) because they are more fluorescent. The third technique states that particles that are small in size enter cells with no further functionalization and the fluorescence property of the molecules may be used for cell imaging applications based on fluorescence.[19] Soot-based methods produce particle mixtures of various colors and isolation of these various colored particles is difficult by gel electrophoresis.[19]

Microwave-assisted method

Guan et al.[20] investigated this method for the development of luminescent C-dots with folic acid molecules as both nitrogen and carbon sources. Initially, a blend was formulated by dissolving folic acid (15 mg) in 3 mL diethylene glycol, and this blend was placed in a domestic microwave oven of 750 W and heated for 40 s. A red-brown-colored suspension was obtained and its dialysis was carried for 3 days against pure water. Luminescent NPs of carbon nitride developed after post treatment, with a size range around 4.51 nm. Under activation at 360 nm, the QY of carbon nitride NPs was found as 18.9%. Even when the emission peak was excited at various wavelengths (from 320 nm to 420 nm), its position remained about constant (at 460 nm). Wang et al.[21] introduced an easy one-stage microwave-assisted method to formulate water-solvent phosphorus-containing C-dots. In this method, a mixture of 2 mL phytic acid (70%) and 1 mL ethylenediamine was prepared with 25 mL ultrapure water, and the resulting turbid mixture was heated for around 8 min in a microwave oven of 700 W. The purification of the crude substance resulted in the development of phosphorus-containing C-dots, also the aromatic structures of these C-dots were covalently attached to phosphorus groups. The phosphorus-containing C-dots indicated two peak emissions when activated at low wavelengths, whereas a single peak was indicated at 525 nm (green fluorescence) by the NPs when activated at high wavelengths (360-460 nm). The QY of the subsequent phosphorus-containing C-dots was 21.65%.[11] In comparison with other approaches, the microwave-assisted method is more convenient and rapidly heats the carbon precursors. This method also simplifies the synthesis process, so the C-dots are readily obtained within a few min with improved QY.[11] Another approach to develop CQDs under this method involves the heating of transparent aqueous solution of PEG_{200} and saccharides in a microwave oven working at 500 W for 2-10 min. When the resultant C-dots are activated at wavelengths extending from 330 to 460 nm, they demonstrate interesting λex-subordinate PL properties. The QY of the C-dots extended from 3.1% to 6.3%. Likewise, C-dots were developed by treating glucose with alkaline (or acidic solution) under ultrasonication for 4 hours. The PL emission of the C-dots covered the whole visible to near-infrared (NIR) spectral region. The C-dots had up-change PL properties when activated at 700-1000 nm, demonstrating outflow in the wavelength range of 450-750 nm.[10]

The hydrothermal and aqueous-based method

In order to develop PL C-dots, used coffee beans were exposed to the hydrothermal treatment. Before grinding into fine powder, used coffee beans were kept in an oven for drying. Later, it was autoclaved and calcined in air at 300 °C for 2 h. C-dots were prepared through four successive stages:

- Dehydration,
- Polymerization,
- Carbonization, and
- Passivation.

Likewise, a green method was conducted to develop C-dots from used green tea leaves at 300 °C for 2 h. The subsequent dark carbonized powder was resuspended in sterile water and later, dialysis was conducted to purify the C-dots. In the development and passivation of C-dots, the rich catechins present in green tea likely played a key role. Four unique molecules [cadaverine, glycine, ethylene diamine-tetra acetic acid (EDTA) 2-amino-2-hydroxymethyl-propane-1,3-diol (TRIS)] comprising either a carboxyl group or an ether group or both in aqueous solvent were independently calcined hydrothermally at 300 °C for 2 h. The outcome revealed that agents with both carboxyl and amino groups were beneficial for the development of highly water-diffusible and photo luminescent C-dots.

Furthermore, in a nitrogen atmosphere, EDTA was used to form C-dots at 400 °C for 2 h. A few EDTA precursors were not entirely degraded, which were then employed to prepare C-dots,

prompting enhanced hydrophilicity. EDTA containing either a carboxylic group or an amino group or containing both in an aqueous solution were individually calcined hydrothermally for 2 h at 300 ˚C. The results showed that precursors that contain both groups i.e. a carboxyl group and an amino group, were beneficial for photoluminescent and highly water-dispersible C-dots.

To develop organosilane-functionalized C-dots, (3-aminopropyl) trimethoxysilane was used as a precursor at 300 ˚C for 2 h, in the absence of an additional passivating agent. Likewise, 4-aminoantipyrine and ammonium citrate was taken as another carbon precursor to develop C-dots in air at 300 ˚C for 2 h. Various organic ammonium species were attached through a covalent bond to the surface, which served as a surface modifier, and altered the hydrophilic nature.[10] A another approach under this method for the development of C-dots, various carbohydrates such as sucrose, glucose, and starch were used in the presence of strong acids such as H_2SO_4 as a starting material. These solutions were further treated with nitric acid and a carboxyl group was introduced on their surfaces to develop a class of carbon nanomaterials, which include C-dots. For further enhancement of their PL intensity, surface passivation with organic molecules and polymers was required.[10] Development of CQDs with glucose as a precursor is easy and simple through a hydrothermal process. To increase the fluorescence emission, ethylene diamine can be used as a passivated agent.[22]

Template method

This approach has also been used for the preparation of nano-sized C-dots. This approach contains two stages:

- Developing C-dots through calcination in the appropriate mesoporous silicon spheres or template, and

- Etching to erase supports and create nano-sized C-dots.

Zong et al.[23] concluded a technique for the use of mesoporous spheres of silica as hard templates. These spheres of silica were saturated with a blended solution of citric acid and complex salts. After mesoporous supports were calcinated and expelled, the photostability of succeeding C-dots along with mono-dispersion demonstrated fabulous luminescence properties. Yang et al.[24] reported a method for developing uniformly morphologic PL C-dots using a soft-hard template approach. Copolymer Pluronic P123 was used in this approach as a soft template, while a hard template requested was mesoporous silica, various organic molecules such as diamine benzene, 1,3,5-trimethylbenzene were sources of carbon. After template removal, passivation, and carbonization, the obtained C-dots' compositions, tunable sizes, and crystalline degrees had additional high stability properties, up-conversion PL and PL effectiveness as high as 3.3-4.7%. The difficulty of aggregate formation was successfully eliminated

Table 1. Properties of different synthetic methods

S no	Synthetic methods	Size range	Quantum yield	Advantages	Disadvantages
1	Arc discharge method	---	-	Most attainable method	Harsh conditions, possess low QY and composite method
2	Laser ablation method	5 nm	QY ranges between 4%-10%	Effortlessness, effective technique, different sized nanoparticles can be prepared	Large amount of carbon matter is required, poor control over sizes, low QY
3	Electrochemical method	6-8 nm	QY ranges between 2.8%-8.9%	Stable method, extent of carbon dots can be managed by changing current density, water-soluble carbon dot can also be prepared	Complex method
4	Thermal route	2-6 nm	QY ranges between 0.1%-3%	Easy and straightforward method, have fluorescence property, appropriate method for particles (on milligram scale)	Low QY
5	Microwave-assisted method	4.51 nm	The PL QY and phosphorus containing QY ranges between 3.1%-6.3% and 21.65% respectively	Simple and convenient method, inexpensive and eco-friendly method	Poor control over sizes
6	Hydrothermal and aqueous-based method	-	-	Highly water-dispersible carbon dots can be prepared, inexpensive, non-toxic	Poor control over sizes
7	Template method	-	-	Carbon dots have biocompatibility and colloidal stability	Time-consuming and expensive method, have limited QY

QY: Quantum yield, PL: Photoluminescence

through this soft-hard template approach, and the development of C-dots was permitted with a narrow distribution of size due to confinement of size. Lai et al.[25] developed C-dots in the NPs of mesoporous silica, which served as a nanoreactor to control the size distribution. Initially the authors developed mesoporous silica NPs ($mSiO_2$); the $mSiO_2$ NPs were blended with PEG-NH_2 and glycerol, then heated to 30 min at 230 °C. Finally, the extraction of crude items was achieved through centrifugation to acquire the C-dot nano-composites without scratching. Simultaneously capping PEG onto the surface of $mSiO_2$, the subsequent C-dots indicated additional improvement in QY, biocompatibility, and colloidal stability. During the synthetic process of C-dots, corrosive acid or base was expected to etch the template because the formation of mesoporous silica was difficult. This strategy was time consuming and expensive. Moreover, the template was hard to be etched off completely due to the high-temperature pyrolysis of the template, also the process of separation and purification was difficult, and the QY was limited.[11] The properties of the different synthetic methods are summarized in Table 1.

Characterization of carbon quantum dots

Keeping in mind the goal to attain information about the synthetic properties of C-dots, numerous techniques may be used in order to characterize C-dots, for example, nuclear magnetic resonance (NMR), X-ray diffraction (XRD), transmission electron microscope (TEM), fourier-transform infrared spectroscopy (FTIR), ultraviolet (UV) spectroscopy, and PL.[11]

TEM

TEM can be used to identify the ultrastructure of samples because it possesses a high resolution of 0.1-0.2 nm. TEM hasa wide demand in science, pharmaceuticals, material science, and other research and development departments. The morphology of NPs can be studied by this technique, in order to understand information regarding their shape, size, and dispersion. TEM is broadly used as a part of the characterization of C-dots. To determine the fine structure of C-dots, high-resolution TEM can also be used. The crystalline nature of C-dots can be classified into two types of lattice fringes, named as interlayer spacing and in-plane lattice spacing, respectively. Interlayer spacing typically is focused at around 0.34 nm, whereas in-plane lattice spacing is focused at 0.24 nm.[11] Zhang et al.[26] carried out acid oxidation of graphite in order to synthesize C-dots and their lattice spacing was generally less than 0.3 nm, demonstrating that the large portion of C-dots were actually separate graphenes. Shinde and Pillai[27] synthesized C-dots from multi-walled carbon nanotube dots by means of an electrochemical technique, and at the same time, two kinds of lattice fringes were observed in the high-resolution TEM image.[11]

XRD

XRD is efficiently used to characterize C-dots and to obtain information of particle size, phase purity, and crystal structure.[11,28] XRD also determines the crystalline phases of CQDs.[29] Liu et al.[30] synthesized C-dots by using hexa-perihexabenzocoronene as the precursor. C-dots with a size of ~60 nm in breadth and 2-3 nm thickness were produced, after pyrolysis at high temperature, surface functionalization, reduction treatment, and oxidative peeling. The so obtained C-dots possessed a fluorescence QY of 3.8%. Mao et al.[31] developed photoluminescent C-dots with glycerol through a one-stage pyrolysis of poly (acrylic acid). The various structures and optical features of the C-dots were altogether examined. The XRD design demonstrated a wide peak near $2\theta=24^{\circ}$, further affirming the white fluorescent C-dots' graphite structure. Bourlinos et al.[2] synthesized C-dots through the calcination of ammonium citrate salt at 300 °C; the relating XRD design showed two reflections that were superimposed, which confirmed the presence of exceptional carbon alkyl groups that were surface modified.[11]

FTIR

For the determination of functional groups that are present on the surface of C-dots, eFTIR has also been used.[10] C-dots mostly comprise oxygen, carbon, and hydrogen. Due to the development of C-dots by the partial oxidation of a carbon precursor, carboxyl or carboxylic acid groups, hydroxyl groups, and ether/epoxy are abundant on the surface of C-dots and so for the investigation of these groups containing oxygen, FTIR is a useful device. Before applying, changes were required to be made with C-dots for balancing out potential wells on the energy surface, lesser cytotoxicity, and higher fluorescence QY. Altered C-dots can be characterized using infrared spectroscopy so as to decide if they are passivated adequately. Peng et al.[32] developed C-dots of size 1-4 nm through the compound oxidation of carbon strands of one micron, 1-4 nm C-dots, the particles so formed broke up in a polar solvent and were soluble in water; dimethyl sulfoxide and dimethyl formamide being examples. The infrared range of these was recorded. Peaks of characteristic absorption at 1724 cm^{-1} and 3307 cm^{-1} proposed carboxyl groups' appearance on their surface; the presence of a double bond was shown by the peak of absorption at 1579 cm^{-1}, and the presence of ether linkage was implied by an absorption peak at 1097 cm^{-1}.[11]

NMR

An NMR strategy is often used to obtain structural information of C-dots. Hybrid tpes of C-atoms in the crystalline network and binding mode between carbon atoms is determined by NMR. Tian et al.[33] used natural gas burning sediment as a carbon source and conducted the refluxing with nitric acid, which resulted in the development of C-dots. Aromatic (sp^2) carbons show resonance in the region extending from 90-180 ppm, whereas aliphatic (sp^3) carbons show resonance in the region extending from 8-80 ppm, structural insights of C-dots is determined with the help of NMR measurements by distinguishing sp^3 carbons from those of sp^2. The absence of aliphatic carbons was indicated by a carbon-13 (13C) NMR range, which depicted the absence of a single peak below 120 ppm. Within the region extending from 120-150 ppm, a sequence of peaks appeared and most of these peaks emerged from aromatic carbons. 13C NMR spectroscopic estimations affirmed that the C-dots had developed from sp^2 carbons.[11]

UV spectroscopy

Strong (UV) absorption is usually shown by C-dots prepared using various techniques, but still the positions of absorption peaks of UV are entirely different for different techniques used for the preparation of C-dots.[12] C-dots of 3.8, 1.5-3, an 1.2 nm transmit at NIR, visible (400-700 nm), and UV (350 nm) regions, respectively.[10] Li et al.[34] added active carbon (4.0 g) into 70 mL of hydrogen peroxide to make a suspension and sonicated it for 2 hours at room temperature. After filtration, fluorescent water-soluble C-dots were obtained with a diameter range of 5-10 nm, and typical absorption of an aromatic pi framework was represented by the common UV-visible absorption band peak at 250-300 nm. Wang et al.[35] immediately added 0.5 g citrus extract anhydrous into N-(β-aminoethyl)-γ-aminopropyl methyl dimethoxy silane solution with vigorous stirring at 240 ˚C and maintained the same for 1 min. Amorphous CNPs of ~0.9-nm diameter were incorporated; after natural cooling and purification, they were also very luminescent (QY=47%). The C-dots thus manufactured had a strong UV-visible absorption peak at 360 nm. Dong et al.[36] used carbonation of citrus acid to form photoluminescent C-dots at 200 ˚C. Their C-dots were nanosheets of 0.5-2.0-nm thickness and ~15 nm in width, demonstrating UV absorption at 362 nm in the absorption range, the NPs were consistent in size and this was evident by the narrow peak width. The maximum emission wavelength remained unaltered at a point when activated at various excitation wavelengths. Tang et al.[37] conducted pyrolysis of a glucose solution assisted by microwave for the preparation of C-dots; the diameter of the obtained C-dots was 1.65 nm with a fluorescence QY of 7-10%. Two evident UV absorption peaks at 228 and 282 nm were indicated by the aqueous solution of these C-dots. The intensity of both UV absorption peaks was increased by extending the microwave heating time, whereas the peak positions remained unaltered and showed no connection with NPs size.[11]

PL

As another class of nanomaterials, C-dots have elicited remarkable consideration in the past decade. From an essential perspective to property and application, PL is the most intriguing characterization of C-dots. C-dots possess certain optical properties that may reflect impacts from particles of various sizes in the sample. In addition to this, various emissive sites are distributed on each C-dot. However, investigations on the optical properties of small-sized C-dots are dubious because the accurate mechanism of PL is unclear. One exceptional feature of the PL of C-dots is the clear λex-dependence of the emission wavelength and intensity. By using surfactant-modified silica spheres as carriers and resols as carbon precursors, C-dots of 1.5~2.5 nm were synthesized followed by surface passivation with PEG1500N. The resulting QY of passivated C-dots was characterized as 14.7%. A suspension of passivated C-dots showed strong blue luminescence when excited at 365 nm. These C-dots have broad emission spectra, extending from 430 to 580 nm, and they exhibit λ-exdependent PL emission. Brilliant and vibrant PL of C-dots can be ascribed to the presence of a surface energy trap settled by surface passivation.[11]

Biodistribution and pharmacokinetics

Subcutaneous (s.c.) and intravenous (i.v.) injections are the basic routes of administration of CQDs. As these QDs reach the systematic circulation, they identify the target and bind to it. After attaching to the target, light is emitted by each QD. The color of the fluorescence depends on the size of the QD and can be easily detected and identified by various techniques. CQDs or C-dots, are small semiconductor nanocrystals of 1-10 nanometers obtained with different surface passivation processes either by modification or functionalization. These have very low toxicity and high fluorescence, and thus have numerous applications in bioanalysis, bioimaging, drug delivery, and other related areas. Accordingly, it is necessary to consider the biosafety studies of C-dots, which includes biodistribution and pharmacokinetics. In 2009, Yang et al.[38] were the first to investigate the biodistribution pattern of C-dots by combining 13C labeling and whole-body imaging. The biodistribution and translocation of C-dots in mice were concluded. It was found that C-dots could not cross the blood brain barrier, but could easily distribute into the whole body. In certain organs such as the spleen, liver, and kidneys, moderate accumulation was observed.[39,40]

Likewise, Yang et al.[38] and Tao et al.[39] conducted toxicity and biodistribution studies by labeling C-dots using ^{125}I. Pharmacokinetic analysis of C-dots was performed using a two-compartment model. The distribution $t_{1/2}$ of C-dots was 0.1 h and the clearance half-life was 2.1 h. The distribution pattern of C-dots was similar to that of Yang et al.'s[38] as moderate accumulation was observed in the spleen, liver, kidney, but not the brain. Biodistribution studies of C-dots were also carried out by Li et al.[34] in mice using i.v. injections. Imaging of dissected and sliced organs was performed under 405 nm excitation and 500 nm emission. Blue fluorescence was detected in different body organs including the spleen, liver, heart, kidney, lungs, small intestine, and brain. High concentrations of C-dots were noticed in the spleen. In other exposure pathways, due to high hydrophilicity and small size, C-dots exhibited free translocation in the body. The biodistribution of Gd-carbon dots was investigated by Xu et al.[41] after intra-tracheal instillation. Gd^{3+} quantification was performed so as to measure the biodistribution of QD. Accumulation of QD in liver, kidney, lungs, heart, and spleen was reported. Tumor-bearing mice had almost the same distribution with an accumulation of 10% injected dose per gram of tissue (ID g^{-1}) in the tumor. The authors stated that C-dots could freely translocate as well as distribute in different organs of the body.[42]

Cytotoxicity

The ability of certain chemicals or mediator cells to destroy living cells is known as cytotoxicity. These mediators or chemicals can induce necrosis (accidental cell death) or apoptosis (programmed cell death) in healthy living cells of humans and animals. C-dots have been considered as possible replacements for organic dyes and metallic quantum in bioimaging due to their chemical stability, broad excitation

ranges, and excellent fluorescence properties. Biocompatibility is considered important for their application in cell labeling and imaging thus making them the most important property of QD. The toxicity of C-dots is a basic concern. Studies related to cytotoxicity of C-dots have been conducted by different scientists, organizations, and institutes, but only a few results and reports are present right now. Zhang et al.[18] investigated the cytotoxicity of carbon QDs on rat mesangial cells. No apparent cytotoxicity and much better biosafety properties of C-dots were reported for biologic fluorescent probe applications. Fluorescent C-dots were synthesized and evaluated for cytotoxicity.[19] Various indicators such as cell viability, malondialdehyde, total reactive oxygen species (ROS), glutathione, and lactate dehydrogenase were evaluated using a human bronchial epithelial (16HBE) cell line. The results showed that C-dots significantly increased the membrane permeability of 16HBE cells. C-dots induce oxidative stress, which exhausts the antioxidant defenses of cells, leading to decreased cell viability. Therefore, surface modification of C-dots could minimize their cytotoxicity.

Sun et al.[14] conducted *in vitro* and *in vivo* cytotoxicity studies of C-dots. Viability, proliferation, and cell mortality of MCF-7 cells (human breast cells) and HT-29 cells (human colorectal adenocarcinoma) were determined by conducting trypan blue and methylthiazole tetrazolium assays after exposing them to C-dots. C-dots thus used were synthesized using PEG1500N laser ablation and surface passivation techniques. C-dots as agents have been employed for *in vivo* testing in mice. They give a bright fluorescent appearance in solution form. No acute toxicologic response was reported when C-dots solutions were injected i.v. in mice. These C-dots were excreted primarily via urine within ~3 h of injection,[38] thus signifying the nontoxic nature of C-dots. Fluorescence of C-dots in the liver and kidney can be observed after 4 h of i.v. injection. High accumulation of carbon dots leads to higher fluorescence in the kidney as compared with that in the liver. Urine being the chief excretion pathway of C-dots, also leads to higher fluorescence. No toxicity was reported when C-dots were administered i.v. in male CD-1 mice. Even exposure of C-dots for 28 days showed no toxic effects.[38] All of the above experiments led to the conclusion that C-dots have extensive adequacy for *in vivo* and *in vitro* bioimaging and drug delivery studies. Different research has suggested that C-dots will have biocompatibility almost equivalent to that of United States Food and Drug Administration-approved organic dyes used in optical imaging e.g., indocyanine green. Although more cytotoxicity studies of C-dots are needed, the data above suggest a bright future for C-dots in drug delivery and bioimaging studies.

Biomedical applications

Bioimaging

CQDs play an important role in biomedical applications; bioimaging is one of the essential applications, which can be defined as the process in which images of living organisms are produced with the help of techniques such as magnetic resonance imaging (MRI), X-rays, and ultrasound. It is also used to determine three dimensional structural information.[43] CQDs have numerous advantages over semiconductor QDs because of their biocompatibility, low toxicity, and strong PL. These properties make CQDs very advantageous in the visualization of biologic systems both *in vivo* and *in vitro*. This is important to know that CQDs in themselves are nontoxic, but it is the passivating agent on the surface of CQDs that is mainly responsible for the cytotoxicity. Surface passivating agents with low toxicity can be safely used for in vivo imaging at higher concentrations. For example, in toxicity evaluations, when PEGylated CQDs were introduced i.v. (8-40 mg kg^{-1} CQD/body weight) into mice for up to 28 days, no significant in vivo toxic effects were observed. When mice were exposed to various doses of CQDs and NaCl control, all physiologic indicators were at the same levels. Therefore, at various exposure levels, CQDs indicated non-toxicity at durations beyond those that can be used for *in vivo* imaging studies.[44] CQDs also possess the fluorescence property with biocompatibility and low biotoxicity. This fluorescence property of CQDs makes them potential candidates for fluorescence bioimaging and multimodal bioimaging both *in vivo* and *in vitro*. For example, a PEGylated CQD was labeled on *E. coli* ATCC25922 and confocal microscopy images were produced at different excitation wavelengths. It was demonstrated that the CQD could be used as a fluorescence contrast agent in mice. An aqueous solution of PEGylated CQDs was injected s.c. into mice and at various excitation wavelengths and fluorescence images were obtained. A noticeable contrast was observed for imaging in both green and red emissions. Similar results were obtained when a similar experiment was conducted on nude mice. More precisely, fluorescence imaging was performed with excitation at various wavelengths ranging from 455 nm to 704 nm along with a s.c. introduction of an aqueous solution of CQD into mice. Excitation at 595 nm showed the best fluorescence contrast.

Another property is multimodal bioimaging, which can be defined as the combination of optical imaging and MRI modalities. MRI demonstrates high spatial resolution and the potential to obtain anatomic and physiologic information. On the other hand, rapid screening was determined by optical imaging. For example, for multi-modality bioimaging, iron oxide-doped CQDs (IO-CQDS) were fabricated. Organic precursors with small Fe_3O_4 NPs (approximate size 6 nm) were thermally decomposed, which led to the formation of IO-CQDS. i.v. injections of IO-CQDS were administered to rats for *in vivo* bioimaging and fluorescence signals appeared in spleen slide samples. The combination of various imaging technologies with the fluorescent imaging of CQDs is also beneficial due to the biocompatibility of CQDs.[12] Loading CQDs with enzyme-responsive mesoporous silica nanocarriers with a pH-switchable zwitterionic surface can be used for targeted imaging and drug delivery to tumors.[45]

Targeted drug delivery

CQDs are one of the most effective carbon-based materials that can be used for various biologic applications due to their biocompatibility. The insignificant cytotoxicity of CQDs makes them potential candidates for safe, effective, and targeted

delivery. CQDs are attractive candidates for theranostic agents, which can be defined as agents that have both therapeutic and diagnostic capabilities. For example, a multifunctional theranostic agent (CD-Oxa) was developed when the surfaces of C-dots containing amine groups were conjugated with an anticancer agent [oxidized oxaliplatin, oxa (IV)-COOH]. CD-Oxa profitably combines the therapeutic properties of Oxa and the optical properties of C-dots. They possess better biocompatibility, bioimaging features, and anticancer effects for *in vitro* studies. The *in vivo* study reveals that distribution of drug can be followed by monitoring the fluorescence signal of CD-Oxa, which assists to customize the dose of medicament along with the injection time. To deliver the DNA to cells, an assembly was prepared by coupling CQDs with gold NPs, followed by conjugation with PEI-pDNA. The experimental study revealed that there was a possible delivery of cells with the aid of CQDs. Delivery of doxorubicinorubicin (anti-cancer) in a multimodality fashion can be possible with the use of CQD-functionalized gold nanorods. Under physiologic and Hixson-Crowell standard conditions, haloperidol (anti-psychotic) grafted CQDs with cysteamine hydrochloride can be used for controlled release for up to 40 h. In addition to bioimaging, conjugation of ciprofloxacin (a broad spectrum antibiotic) with CQDs under physiologic conditions also gave an efficient new nanocarrier for controlled drug release.[12,44,45]

Nanomedicine

CQDs, being small fluorescent NPs, serve as a better alternative to other fluorescent nanomaterials. CQDs have an appreciable application in nanomedicine because they do not cause any kind of toxicity in animals. In an experiment, CQDs were injected i.v. in mice and an evaluation was conducted after 4 weeks, which concluded that there was no significant effect on organs and their internal functions. Their insignificant effect and low cytotoxicity level allow them to be used for *in vivo* studies. In plasma samples, highly biocompatible CQDs supported by prothrombin time assays concluded that CQDs did not affect thrombin activity. Also, they did not lead to coagulation of the blood. CQDs have an attractive application in photodynamic therapy (therapy that uses special drugs that activate by light for the treatment of superficial tumors). Cancer cells MCF-7 and MDA-MB-231 are effectively inhibited by CQDs. In addition, CQDs are promising photosensitizers because they are able to produce ROS and the selective localization of CQDs into tumors makes them suitable candidates for photosensitization.

Route of administration and surface coating are both factors that influence the circulation and uptake of CQDs. There is rapid and effective excretion of CQDs from the body when they are administered through the parental route (i.v., intramuscular and s.c. injection). In photodynamic therapy, the up-conversion property of CQDs plays an important role in the treatment of deep-seated tumors; conjugation of CQDs with protoporphyrin IX (a conventional photosensitizer) followed by the indirect excitation (800 nm) of photosensitizer via Forster/fluorescence resonance energy transfer (FRET). Excitation at 800 nm comes under the phototherapeutic window and is able to penetrate four times deeper into human tissue as compared with excitation with light at 630 nm, which has been used in clinical photodynamic therapy. CQDs also play a significant role in radiotherapy; the coating of a silver shell (C-Ag-PEG CQDs) on PEG-CQDs makes them accessible to be used as radiosensitizers in Du145 cells. The availability of CQDs as nanocarriers makes them efficient for tracking and the delivery of genes or drugs, branched polyethylenimine CQDs possess considerable potential for gene delivery. CQDs also have an appreciable application in controlled drug release-controlled release of drug in HeLa cells can be achieved by loading CQDs with doxorubicin. However, it is not clear whether CQDs can specifically target a disease state, thus limiting their efficacy in therapeutic applications.[12,44,45]

Biosensing

CQDs have a wide application in biosensing. Certain properties of CQDs, such as high water solubility, surface modification flexibility, better cell permeation, low toxicity, and high biocompatibility make them potential biosensors. Cellulose, copper, glucose, nucleic acid, iron, potassium, an phosphate can be monitored visually with the aid of CQD-based biosensors. CQDs can be used as a successful fluorescent sensing agent for the detection of nucleic acid with a selective single-base mismatch. The idea involves the adsorption of fluorescently-labeled single-stranded DNA (ssDNA) via CQDs by means of pi-pi association, which was accustomed by extensive fluorescence quenching, further hybridized with its target to consequence for the formation of double sDNA. As a result, it was observed that the ssDNA was desorbed from the surface of CQDs, which was accompanied by a successive revival of fluorescence, prying with the target DNA. Detection and imaging of mitochondrial H_2O_2 were verified by means of CQD-based FRET. CQDs fill in as a contributor of energy transfer and transporter for the sensing framework. Covalently-linked CQDs with an H_2O_2 recognition element, boronate-secured fluorescein can be used for the imaging of H_2O_2, which was endogenously produced in RAW 264.7 macrophage cells.[12] The principle of FRET and homogenous assays was employed to develop an immunosensor for 4,4-dibrominated biphenyl (PBB15) detection-an organic pollutant that causes endocrine system disturbance. The immunosensor consists of antibodies of PBB15, functionalized with gold NPs (AuNP) that behave as the fluorescence acceptor, and PBB15 antigens labeled with carbon QDs, which behave as a fluorescence donor. FRET resulted that CQD fluorescence was efficiently quenched by means of AuNP. On adding PBB15 to the solution, competitive immunoreactions took place and antigens labeled with CQDs were unconfined from the surface of AuNP and resulted in fluorescence recovery. This particular immunosensor was used as a fine example for the immunoassay development to identify analytes with preferable antigens and antibodies.[12,44,45] Vitamin B_{12}-coated CQD serve as a radiometric nanosensor that exhibit an excellent selectivity for phenolic carbofuran.[46] In addition to this, CQDs are used as a fluorescent probe for recognizing small bioanalytes (anti-bacterial drugs). An experimental study was conducted on such example, which involves the

Table 2. Different applications of delivery systems

S No	Delivery system	Applications	References
1	Carboxylic functionalized CQD	For DNA detection and fabrication of DNA biosensor	47
2	Photoluminscent CQD	For Fe^{3+} detection in biosystems as a biosensor reagent	48
3	CQD	For multiphoton bioimaging	49
4		For optical bioimaging *in vivo* and *in vitro*	50
5		For photocatalytic energy conversion as photocatalyst	51
6	CQD loaded with mesoporous silica nanocarriers	For targeted drug delivery and imaging to tumor	45
7	Carbon nanotubes	For treatment of tumors	52
8		For chemotherapeutic drug delivery	52
9		For *in vivo* cancer therapy as drug delivery agent	52

CQD: Carbon quantum dots

production of fluorescent N-CQDs from glutamic acid. These N-CQDs were produced by a one-step pyrolysis technique. The resulting N-CQDs were further used for amoxicillin (antibacterial) detection. Other small bioanalytes such as ascorbic acid, dopamine, and glucose were also detected by CQDs.[12,44,45]

Photocatalysis

Recently, the process of photocatalysis has increased with colossal force as greener options in natural synthesis. Awareness in the photocatalytic process has been roused to some degree by the acknowledgment that sunlight is an adequate unlimited source of energy. Nonetheless, the elevated vitality of UV and short wavelength visible light can unfavorably harm natural compounds. The exhibited capacity of outfitting the extended wavelength light and vitality conversion with a CQD solution offers a fantastic opportunity for their use as photocatalysts in the natural synthetic process. A recent review has shown that CQDs in the range of 1-4 nm are powerful NIR light-determined photocatalysts for specifically oxidizing alcohols into benzaldehydes with great conversion proficiency (92%) and specificity (100%) because of their incredible catalytic action for H_2O_2 deterioration and NIR light-determined electron transfer activity. Doping of 171 CQDs and fitting the surface groups can adequately modulate the photocatalytic activity of CQDs. In contrast, CQDs in the range of 5-10 nm combined via electrochemical removal of graphite indicated light-instigated proton features in solution form, which can serve as an acid catalyst for catalyzing a sequence of natural changes in watery mediums under visible light. As a standout amongst the most well-known photocatalysts, TiO_2 has been used as a part of the expulsion of natural contaminations and for generating H_2 via water splitting. However, a noteworthy downside in its photocatalytic proficiency dwells in its insufficient use of visible light as the illumination source. Just under 5% of sunlight is used by TiO_2 because a bandgap of bulk TiO_2 comes under the UV area (3.0-3.2 eV). Therefore, bandgap engineering through a conceivable change of TiO_2-based media is one of the conceivable ways to deal with upgrading the execution of TiO_2 photocatalysts. In perspective of their appealing optical properties and up-change specifically, a nanocomposite of TiO_2 and CQDs is required to understand the productive use of the full sunlight spectrum. By using methylene blue (MB) as the model agent, it has been demonstrated that nanocomposites of CQDs and TiO_2 can totally deteriorate MB (50 mg/mL) under visible light illumination in 25 min, where just 0.5% of MB is debased when immaculate TiO_2 is used as a photocatalyst. Apart from gathering visible light and changing it over to short wavelength light by up-transformation, which thusly energizes TiO_2 to frame pairs of electron-hole, it is trusted that the nanocomposites of CQDs encourage the relocation of electrons from TiO_2 and the electrons can carry unreservedly along the directing ways of the CQDs, permitting charge partition, adjustment and obstructing recombination, and accordingly creating extensive openings (holes) at the TiO_2 surface. Long-lived openings then record for the greatly improved photocatalytic action of the CQD-TiO_2 nanocomposites. In a like manner, comparative conduct was seen with TiO_2-CQD nanotube composites TiO_2-CQD nanosheet nanocomposites and TiO_2-CQD nanotube composites in the photocatalytic deterioration of rhodamine and MB respectively.[12,44,46] Different applications of various delivery systems are summarized in Table 2.

CONCLUSIONS

- In this review, we have elaborated the recent advancement in CQDs, emphasizing their synthesis methods and characterization, followed by their biomedical applications.

- The unique properties of CQDs are beneficial for potential applications in biomedical science and research.

- C-dots have extensive adequacy for *in vivo* and *in vitro* bioimaging and drug delivery studies.

- Because of their biocompatibility, low toxicity, strong PL, synthetic and photograph steadiness, C-dots have become a fascinating material for bioimaging and in the detection of different analytes.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Dr. Madhu Chitkara (Vice Chancellor, Chitkara University, Rajpura, Punjab, India) and Dr. Sandeep Arora (Dean, Chitkara University, Rajpura, Punjab, India) for support and institutional facilities.

Conflict of interest: No conflict of interest was declared by the authors.

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