İstanbul Journal of Pharmacy

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İstanbul Journal of Pharmacy

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AIMS AND SCOPE

İstanbul Journal of Pharmacy (Istanbul J Pharm) is an international, scientific, open access periodical published in accordance with independent, unbiased, and double-blinded peer-review principles. The journal is the official publication of İstanbul University Faculty of Pharmacy and it is published triannually on April, August, and December. The publication language of the journal is English.

İstanbul Journal of Pharmacy (Istanbul J Pharm) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of pharmaceutical sciences. The journal publishes original articles, short reports, letters to the editor and reviews.

The target audience of the journal includes specialists and professionals working and interested in all disciplines of pharmaceutical, also medicinal, biological and chemical sciences.

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All expenses of the journal are covered by the İstanbul University.

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INSTRUCTIONS TO AUTHORS

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After plagiarism check, the eligible ones are evaluated by the editors-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope. The editor provides a fair double-blind peer review of the submitted articles and hands over the papers matching the formal rules to at least two national/international referees for evaluation and gives green light for publication upon modification by the authors in accordance with the referees' claims.

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Only those manuscripts approved by its every individual author and that were not published before in or sent to another journal, are accepted for evaluation.

Submitted manuscripts that pass preliminary control are scanned for plagiarism using iThenticate software. After plagiarism check, the eligible ones are evaluated by editor-in-chief for their originality, methodology, the importance of the subject covered and compliance with the journal scope.

The selected manuscripts are sent to at least two national/international referees for evaluation and publication decision is given by editor-in-chief upon modification by the authors in accordance with the referees' claims.

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Editor-in-Chief does not allow any conflicts of interest between the authors, editors and reviewers. Only he has the full authority to assign a reviewer and is responsible for final decision for publication of the manuscripts in the Journal.

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An approval of research protocols by the Ethics Committee in accordance with international agreements (World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects," amended in October 2013, www.wma.net) is required for experimental, clinical, and drug studies. If required, ethics committee reports or an equivalent official document will be requested from the authors. For manuscripts concerning experimental research on humans, a statement should be included that shows that written informed consent of patients and volunteers was obtained following a detailed explanation of the procedures that they may undergo. For studies carried out on animals, the measures taken to prevent pain and suffering of the animals should be stated clearly. Information on patient consent, the name of the ethics committee, and the ethics committee approval number should also be stated in the Materials and Methods section of the manuscript. It is the authors' responsibility to carefully protect the patients' anonymity. For photographs that may reveal the identity of the patients, signed releases of the patient or of their legal representative should be enclosed.

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- Copyright Agreement Form
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Keywords: Each submission must be accompanied by a minimum of three to a maximum of six keywords for subject indexing at the end of the abstract. The keywords should be listed in full without abbreviations. The keywords should be selected from the National Library of Medicine, Medical Subject Headings database (https://www.nlm.nih.gov/mesh/MBrowser.html).

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Original Articles: This is the most important type of article since it provides new information based on original research. The main text of original articles should be structured with Introduction, Materials and Methods, Results, Discussion, and Conclusion subheadings. Results and Discussion sections can be combined under "Result and Discussion" heading. Please check Table 1 for the limitations for Original Articles.

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Short Papers: Please check Table 1 for the limitations for Short Papers.

Letters to the Editor: This type of manuscript discusses important parts, overlooked aspects, or lacking parts of a previously published article. Articles on subjects within the scope of the journal that might attract the readers' attention, particularly educative cases, may also be submitted in the form of a "Letter to the Editor." Readers can also present their comments on the published manuscripts in the form of a "Letter to the Editor." Abstract, Keywords, and Tables, Figures, Images, and other media should not be included. The text should be unstructured. The manuscript that is being commented on must be properly cited within this manuscript.

Tables

Tables should be included in the main document, presented after the reference list, and they should be numbered consecutively in the order they are referred to within the main text. A descriptive title must be placed above the tables. Abbreviations used in the tables should be defined below the tables by footnotes (even if they are defined within the main text). Tables should be created using the "insert table" command of the word processing software and they should be arranged clearly to provide easy reading. Data presented in the tables should not be a repetition of the data presented within the main text but should be supporting the main text.

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All acronyms, abbreviations, and symbols used in the manuscript must follow international rules and should be defined at first use, both in the abstract and in the

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Original Article	3500	250 (Structured)	6	7 or total of 15 images
Review Article	5000	250 (Unstructured)	6	10 or total of 20 images
Short Paper	1000	200	No tables	10 or total of 20 images
Letter to the Editor	500	No abstract	No tables	No media

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When submitting a revised version of a paper, the author must submit a detailed "Response to the reviewers" that states point by point how each issue raised by the reviewers has been covered and where it can be found (each reviewer's comment, followed by the author's reply and line numbers where the changes have been made) as well as an annotated copy of the main document. Revised manuscripts must be submitted within 30 days from the date of the decision letter. If the revised version of the manuscript is not submitted within the allocated time, the revision option may be cancelled. If the submitting author(s) believe that additional time is required, they should request this extension before the initial 30-day period is over.

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Original Article

Development of liposomal topical gel of bexarotene for effective management of cutaneous t-cell lymphoma: Formulation to preclinical assessment

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ABSTRACT

Background and Aims: The objective of this work was to formulate liposomal gel formulation of bexarotene for Cutaneous T-cell Lymphoma (CTCL). Low solubility and high log P value make the drug a poor candidate for its penetrability and absorption through the transdermal route. When bexarotene is incorporated into liposomal formulation, its solubility and permeability can enhance.

Methods: In the present investigation, the liposomes of bexarotene were prepared by thin film-hydration method and optimized for different critical processing parameters such as the amount of lipid and the time of stirring. Checkpoint batches were prepared to validate the mathematical model.

Results: The final optimized liposome formulation, which has more than 85% entrapment efficiency and vesicle size of 625 nm, was prepared. The optimized liposomes were loaded (equivalent to 1% w/w bexarotene) into the carbopol gel (1.5% w/w) and, evaluated for physico-chemical parameters. In vitro drug permeation and deposition of promised liposomal gel were performed through rat skin. The skin irritation studies of the liposomal gel were examined on rats, in vivo. MTT assay was performed to determine the cytotoxicity and cell apoptosis on CTCL specified cell line (Hut-78) by bexarotene liposomal gel. The optimized liposomes of bexarotene (FL1) were found to be spherical having a vesicle size of 639 nm with PDI 0.115 and a zeta potential value of -19.3 mV. The promised liposomal gel (LG5) evaluations were found in the limit. The LG5 was shown 31% bexarotene deposition in the skin. The experiment revealed a significant decrease ($p<0.005$) in the number of viable cells following MTT assay.

Conclusion: The liposomal gel formulation of bexarotene improved the treatment and management of CTCL.

Keywords: Bexarotene, Liposome, Topical Gel, Lymphoma, MTT assay, UV skin irritation studies

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INTRODUCTION

The cutaneous T-cell lymphoma (CTCL) is the most common form of non-Hodgkin's lymphoma that involves skin, blood, lymph nodes, and other internal organs (Olsen, 2015). In the early and advanced stages of the disease, the symptoms are observed only in the skin, including itching, dryness, rashes, and enlargement of lymph nodes. Various epidemiological studies have substantiated that CTCL disease is also promoted or triggered by environmental and external exposures, viz., air pollution and chemical exposure, including pesticides, detergents, and UV rays (Zinzani et al., 2016).

Bexarotene is a highly effective anticancer agent that has been approved for the treatment of CTCL. Bexarotene, a third-generation selective retinoid X receptor agonist, triggers the receptor and initiates prompting cell variation, which has been shown to demonstrate promising effects in the management and treatment of CTCL. The mechanism of action of bexarotene is not completely understood. Recent studies reported that the induction of apoptosis in CTCL cell lines (MJ, Hut78, and HH; IC_{50} ~160-171mM) in association with activation of caspase-3 and cleavage of poly–adenosine diphosphate-ribose polymerase and interleukin-4 production, which plays a major role in the systemic immunosuppression that is characterized by advancing Sézary syndrome, may be inhibited by bexarotene (Chen et al., 2014). However, the topical delivery of bexarotene is diminished due to its high log P (6.9) value and poor aqueous solubility, therefore, it is difficult to penetrate the different layers of skin (Agarwal, Katare, & Vyas, 2001). Liposomal delivery of bexarotene can improve the transdermal delivery and increase cell membrane penetration as well as the treatment of cutaneous T-cell lymphoma.

Liposomes are spherically-shaped and nano-structured vesicles composed of an outer bilayer envelope made up of one or more non-toxic, biodegradable, and biocompatible phospholipids with an aqueous core that easily entraps both hydrophobic and hydrophilic therapeutic agents. They are proven for the effective delivery of the therapeutic agents topically for various disorders of the skin as (Sharma & Verma 2017; Minh et al., 2015). The present research aims to develop a liposomal topical gel of bexarotene and characterize it for various physicochemical parameters followed by skin irritation studies and *in vitro* cytotoxicity activity (Hashemi, Karami-Tehrani, & Ghavami, 2004).

MATERIALS AND METHODS

Materials

Bexarotene was received as a gift sample from Apicure Pharmaceuticals Pvt. Ltd. (Vadodara, India); Phospholipon 90H (PL-90H), Phospholipon 100S (PL-100S), and Soya phosphatidylcholine (SPC) were purchased from Himedia (Mumbai, India), Cholesterol from Lobachem (India), and ethanol and ethyl acetate from SD Fine Chemicals Ltd. (India). Carbopol 934P was acquired as gift sample from Lubrizol Limited (India). Ethanol was purchased from Finar Pvt. Limited (India). Cellophane membrane (MW 12000–14000) was purchased from Himedia, (India); methanol and other chemicals used were of analytical grades.

Development of liposomes

Liposomes of bexarotene were prepared by the thin-film hydration method using a rotary evaporator (Heidolph Hei VAP Advantage AQ/G3, Germany). The drug was solubilized in a blend of ethyl acetate and ethanol in the ratio of 1.5:1.0. The thin film formed by using a mechanical dispersion of bexarotene, different phospholipids like phospholipon 90H, phospholipon 100S, soya phosphatidylcholine (1-4% w/v), and cholesterol (1% w/v) was appropriately mixed in ethyl acetate in a 250 mL round bottom flask (RBF) as given in Table 1. By using the rotary evaporator in reduced pressure, organic solvents were condensed and recovered, which created a thin film around the inner surface of the flask at 50ºC and 90 rpm. The obtained film was subjected to vacuum for an hour for the complete removal of the remaining solvent. Then, the dried film was hydrated with a phosphate buffer (pH 7.4) for 30 min. The ready mixture was vortexed and sonicated in a bath sonicator (Ultra Sonic, India) for 15 min. and the solution of liposome was prepared and stored in the refrigerator for further evaluations (Akbari et al., 2020).

Characterization of liposomes

Vesicle size determination

The dynamic light scattering method was employed to determine the vesicle size of bexarotene liposomes. The liposomal suspension was diluted with water (10 times, MilliQ®) and transferred in the zeta sizer cell (Malvern Zetasizer NanoZS90, UK), and the vesicle size was determined according to the standard operating procedure. The temperature of the cell was kept constant at 25°C and three values were obtained at 175°scattering angle (Manosroi, Kongkaneramit, & Manosroi, 2004).

Zeta potential determination

Polydispersity index was determined by zeta potential, which could be a measure of uniformity. The sample for determining the zeta potential was prepared similarly as that for the determination of vesicle size. It was then examined thrice from -200 mV to +200mV as per the method of Dicko *et al.,* 2010.

Morphological evaluation

The surface morphology of liposomes was evaluated using transmission electron microscopy (TEM) by JEM 2100 microscope (Hitachi, H-7650, Japan). The diluted liposomal dispersion was fixed on the copper grids (Formvar®) for morphological studies. The diluted liposomal suspension was stained with the solution of uranyl acetate (2%, w/v) for proper imaging of samples (Fathalla Youssef, & Soliman., 2020).

Entrapment efficiency and drug loading

For determination of entrapment efficiency (%, EE) the ultrafiltration centrifugation method was applied, wherein 2 mL of the formulation was centrifuged at the rate of 10.000 rpm for 30 minutes utilizing a cooling centrifuge AmiconUltra-4 (Ultracel-10) centrifugal filter (Merck Millipore Ltd., Germany) (Fathalla et al., 2020). Both pellet and supernatants were collected and the amount of free drug within the supernatant liquid was determined utilizing the RP-HPLC assay method. The entrapment efficiency in liposomes was calculated as

$$\% \, Entrapment \, efficiency = \frac{Ct - Cr}{Ct} \times 100$$

Ct = Total drug concentration, Cr = Free drug concentration.

In vitro drug release studies
The dialysis method was employed to study the in vitro drug release from liposomes. Ten milliliters of the bexarotene liposomes were placed in a dialysis bag (Spectra Por S/P 2 dialysis membrane, 12K–14K molecular weight) and dipped in 40 mL dialysis media (phosphate buffer, pH 7.4 with Tween 80 (0.02%, v/v) to maintain sink condition at 37±0.5°C, and stirring rate was kept constant at 50 rpm. Samples of two milliliters of release medium were withdrawn for analysis at one-hour intervals and replaced with a fresh medium. Drug release studies were continued for 12 hours, and the obtained samples were analyzed using the RP-HPLC method (Singh et al., 2005).

Optimization of liposomes
In the preliminary studies, various lipids were screened out for the preparation of bexarotene liposomes viz., PL-100S, PL-90H, and SPC, respectively, and sonication time, cholesterol-lipid ratio, and drug loading during the experiment were studied. Bexarotene liposomes were optimized using Response Surface Methodology by 3^2 full factorial design methods. The two factors at three-level design were employed for constructing second-order polynomial models (quadratic) using Design Expert 11 (Version Trial; Stat-Ease Inc, Minneapolis, Minnesota) software. A total of nine formulations, as shown in Table 2, were prepared according to experimental design. The selected independent variables were the amount of phospholipon-100S (X1) and time for rotation (X2). The entrapment efficiency (%) and vesicle size (nm) were selected as dependent variables (Garg et al., 2017).

The responses were calculated using interactive and polynomial terms of the mathematical model.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1X_1 + b_{22}X_2X_2 + b_{12}X_1X_2$$

In the equation, Y denotes the dependent variable, b_0 is the arithmetic mean b_1, and b_2 are the coefficients for the factors X_1 and X_2. Optimized formulation was validated, and analysis of variance (ANOVA) was used to analyze the results. ANOVA was applied to find out the significance of factors (Jangde & Singh, 2016).

Checkpoint batches
The checkpoint batches were prepared and compared with values obtained from the software utilizing the equations of the model validity. Checkpoint batches were prepared and evaluated for percent entrapment efficiency and vesicle size. The predicted value and observed values were compared and residual values were determined (Table 3).

Optimized batch
Final optimized Bexarotene liposome batch FL1 was prepared as per the suggested quantity of X1 and X2 factors, which was set targeted for entrapment efficiency (85%) and vesicle size (625 nm), as shown in Table 3. The method of preparation of the liposome was the same as that mentioned previously.

Preparation of liposomal topical gel
For the preparation of the liposomal topical gel, firstly the Carbopol 934P (0.5 to 1.75%, w/w) was splashed into distilled water (Table 4). The topical gel was formed by swelling and hydration carbomer for 3-4 h. The acidic nature of Carbopol suspension was neutralized using triethanolamine for crosslinking and gel formation. Then isopropyl alcohol, methyl paraben, and propyl paraben were added and blended with a gel base. The liposomal suspension (FL1) was at that point blended with this Carbopol gel base by mechanical blending at a speed of 1000-1500 rpm for 1 h to make the liposomal topical gel dispersion (Yu et al., 2019).

Characterization of liposomal topical gel
The developed liposomal topical gels were characterized physically for their color, transparency, odor, morphology, and

Table 1. Development of bexarotene liposomes.

Form. Code	Lipid (%, w/w)			Cholesterol (%, w/w)	Drug (%, w/w)	Visual Appearance	Vesicle Size (nm)	EE (%)	PDI
	PL-90H	SPC	PL-100S						
L1	1	-	-	1	1	Rough layer	835.5±5.4	61.1±2.1	0.519
L2	2	-	-	1	1	Rough layer	901.3±6.8	72.3±2.8	0.573
L3	3	-	-	1	1	Rough layer	983.5±8.2	82.1±3.8	0.641
L4	4	-	-	1	1	Rough layer	995.4±9.3	73.3±6.5	0.654
L5	-	1	-	1	1	Rough layer	779.3±3.7	61.1±1.9	0.522
L6	-	2	-	1	1	Rough layer	895.4±4.8	69.3±2.8	0.571
L7	-	3	-	1	1	Rough layer	885.4±8.3	71.2±2.5	0.481
L8	-	4	-	1	1	Rough layer	991.4±7.4	83.5±2.6	0.594
L9	-	-	1	1	1	Smooth layer	182.4±2.4	65.1±1.5	0.123
L10	-	-	2	1	1	Smooth layer	213.2±2.9	81.2±1.8	0.271
L11	-	-	3	1	1	Smooth layer	639.2±3.6	89.9±1.9	0.116
L12	-	-	4	1	1	Smooth layer	715.2±3.7	74.2±2.4	0.317

Table 2. Design matrix 3^2 full factorial design, ANOVA responses, and the observed coefficient for optimization of bexarotene liposomes.

Batch	X1: Amount of Phospholipon-100S (%w/w)	X2: Time of Rotation (Minutes)	Y1 Entrapment Efficiency (%)	Y1 Vesicle Size (nm)
OL1	-1	-1	60.34±1.33	170.92±10.32
OL2	-1	0	65.13±1.24	182.45±10.56
OL3	-1	+1	63.16±2.23	176.84±15.52
OL4	0	-1	78.34±2.34	242.56±16.25
OL5	0	0	81.25±2.48	213.24±13.33
OL6	0	+1	79.46±2.89	225.28±25.49
OL7	+1	-1	86.52±3.79	620.46±16.49
OL8	+1	0	89.96±2.32	639.27±18.33
OL9	+1	+1	90.56±3.49	656.59±25.33
Coded Value		**-1 (Low)**	**0 (Intermediate)**	**+1 (High)**
X1	Amount of Phospholipon-100S (%, w/w)	1	2	3
X2	Time of Rotation (Minutes)	10	15	20
Independent Variables	**Y1**		**Y2**	
	Entrapment Efficiency (%)		**Vesicle Size (mm)**	
		ANOVA Response		
F Value	20.71		188.24	
P Value	0.0156		0.0006	
R^2	0.9718		0.9968	
Adjusted R^2	0.9249		0.9915	
Predicted R^2	0.6687		0.9663	
Adeq Precision	14.13		39.2413	
		Coefficients		
b_0	73.222		376.333	
b_1	9.833		196	
b_2	-10.833		76.5	
b_{12}	-0.25		20	
b_{11}	2.166		49	
b_{22}	-0.833		-12.5	

separation of phases. The pH of the prepared liposomal topical gels was determined using a digital pH meter (Benchtop pH meter, Mettler Toledo, Mumbai). The topical gel was dispersed in milliQ water, and the electrode of pH meter was plunged inside the topical gel container and the pH was recorded until a constant reading was achieved. The parallel plate method was used to determine the spreadability of the liposomal topical gel. The liposomal topical gel (100 mg) was put on a transparent glass slide and another transparent slide of glass was put upon the slide containing the topical gel. Initially, the spread topical gel diameter was recorded by the weight of the glass slide only. After stress given by weight (ranging from 5 g to 100 g for a 30-second interval) on the liposomal topical gel, the spread diameter was calculated after the addition of each weight (Bavarsad et al., 2016). The spreadability of the topical gel was determined by the following equation:

$$S = m\,X\,\frac{l}{t}$$

where, S = spreadability, m = weight on upper slides, l = glass slide length (7.5 cm), t = time (s).

The viscosity of the liposomal topical gel was determined with the help of a Brookfield viscometer (S-62, LVDV-E) at 25 ºC with a fixed speed of 12 rpm. Drug content of the liposomal topical

Table 3. Checkpoint batches and optimized batch.

Form. Code	Lipid (% w/w) Phospholipon 100S	Rotation Time (minutes)	Entrapment Efficiency (%)			Vesicle Size (nm)		
			Predicted Valve	Observed Value	% Residual Value	Predicted Valve	Observed Value	% Residual Value
LC1	1.5	12.5	73.99	74.23±12.35	0.32	254.20	248.12±12.39	2.45
LC2	1.5	17.5	63.28	61.26±6.31	3.29	320.78	333.10±18.21	3.69
LC3	2.5	12.5	83.95	86.91±11.25	3.40	440.20	448.31±15.28	1.80
LC4	2.5	17.5	72.99	75.84±10.56	3.75	526.70	551.45±19.84	4.48
FL1	3	15.5	84.99	89.9±1.9	4.67	623.26	639.2±4.9	2.49

Table 4. Characterization of liposomal topical gel formulations.

Code	Polymer (%, w/w)	Formulation Incorporated/Drug	Appearance	Viscosity (cPs)	Drug Content (mg)	Spreadability (cm)	pH
G1	1.5	Pure Drug	Loose, Non-greasy, Homogenous	4332.2	99.1±2.6	4.5±0.55	6.9±0.2
LG1	0.5	FL1	Loose, Non-greasy, Homogenous	1291.6	95.3±1.9	5.4±0.22	6.7±0.1
LG2	0.75	FL1	Non-greasy, Homogenous	1869.5	97.3±1.3	5.3±0.35	6.9±0.3
LG3	1.0	FL1	Non-greasy, Homogenous	2494.7	94.6±2.4	5.0±0.51	6.9±0.1
LG4	1.25	FL1	Non-greasy, Homogenous	3196.8	96.5±1.9	4.7±0.52	6.7±0.3
LG5	1.5	FL1	Non-greasy, Homogenous	4286.9	93.7±1.4	4.6±0.24	6.8±0.2
LG6	1.75	FL1	Hard, Non-greasy, Heterogeneous	5619.7	97.2±1.9	3.8±0.68	6.9±0.4

Table 5. Skin irritation data.

Group	Treatment	Dose (mg/cm^2)	No. of Animals	Wrinkle Score
Group I	Control	-	06	0
Group II	UV treated + Drug free gel topically	50	06	$2.83±0.408^{NS}$
Group III	UV treated + Drug free Liposomal gel topically	50	06	$2.33±0.516^{NS}$
Group IV	UV treated + Drug Liposomal gel topically	50	06	$2.28±0.753^{NS}$

Values are expressed as Mean±SD One-way Anova followed by Bonferroni test,
*P<0.050 significant and NSP<0.050 non-significant compared to the UV treated group.

gel was estimated by shaken 100 mg bexarotene equivalent liposomal topical gel with an adequate quantity of methanol to extract the bexarotene and then estimated by utilizing the RP-HPLC method. The *in vitro* diffusion of the drug from the liposomal topical gel and plain drug-loaded topical gel was examined utilizing a Franz diffusion cell apparatus kept at 37±1^{0}C. The actual diffusion area diameter of the diffusion cell apparatus was 2.30 cm^2. The receptor compartment consisted of 6.5 mL phosphate buffer (pH 7.4) and was stirred at 100 rpm. Cellophane membrane (MW; 12K–14K Dalton) was fixed between the compartments of the donor and the receptor. The formulation was placed in the donor compartment. The

aliquots were taken from the Franz diffusion cell at different time intervals and after each sampling, the diffusion media already kept at 37±1^{0}C was exchanged into the receptor compartment (Priprem et al., 2018).

Skin permeation studies

The shaved dorsal skin of sacrificed albino rats was used for diffusion studies. The fat was separated from the near dermis side using a blade and alcohol. Collected skin was cautiously washed with phosphate buffered saline solution (PBS, pH 7.4) and stored in the deep freezer (-20°C). Before initiating diffusion studies, the diffusion medium (PBS pH 7.4 containing Tween® 80 (0.02%, v/v) to maintain sink condition) was added to the Franz diffusion cell and kept at 37±1^{0}C for one hour. The optimized liposomal topical gel was applied on dorsal skin. Aliquots were withdrawn at different time intervals. The amount of the receptor fluid was kept constant by introducing a new receptor fluid sample. The RP-HPLC method was used to analyze the samples (Surini, Leonyza & Suh, 2020).

Drug deposition studies

The rodent skin which was used in the skin permeation studies was carefully expelled. The skin was washed with the mixture of methanol and distilled water (1:1) to eliminate traces of the drug formulation on the outer part of the skin portion. The washed skin was divided into small portions by cutting and dipped in a 10 mL volume of 0.05% trypsin solution. Sample tests were put on a mechanical stirrer at 100 rpm and at 37±1^{0}C for 24 h. Samples were analyzed after filtration using the RP-HPLC method.

Skin-irritation studies

The designed protocol for the animal studies was approved by IAEC (PBRI/IAEC/PN-19016). The animals were subdivided into 4 groups of 24 animals. Animals of Group I served as the control group, while Animals of Group II received five-minute ultraviolet light exposure two times a day and served as the irradiated control group; drug-free topical gel was applied. The test groups III and IV received both ultraviolet radiation and drug-free liposomal topical gel and bexarotene liposomal topical gel treatment, respectively. The treatment was provided to animals four hours before ultraviolet radiation exposure as per the protocol guidelines. All the animals were kept in a locally prepared wooden ultraviolet simulator or chamber. The ultraviolet lamp (300W Waton® bulb, Germany) was fixed inside on the top of the chamber so the animals received radiation from a distance of 40 cm. The bulb produces the full spectrum of ultraviolet radiation from 260 - 400 nm. Ultraviolet radiation exposure was controlled by exposure time. The animals were treated for a month to study the changes obtained on dorsal skin (Jewett *et al.* 2020; Raimondi, Suppa & Gandini, 2020).

In-vitro cell proliferation studies

A well-developed MTT assay was used to assess the proliferative ability of cells treated with bexarotene-loaded topical gel and bexarotene-loaded liposomal topical gel. Around 5×10^3 Hut78 cells *per* well were inoculated in 96-well formats. Serum medium RPMI was changed to serum-free RPMI medium after a one-day incubation period (Madan et al., 2012). After completion of a day, cells were treated with bexarotene-loaded topical gel and bexarotene-loaded liposomal topical gel at gradient concentrations ranging from 0-150µM/mL in PBS (pH 7.4) for three days. At the end of treatment, 5 mg/mL MTT was added to each well, and the plate was incubated at 37°C in the dark for 4 h. The formazan product was then dissolved in 100 µL of DMSO after removing the medium or content of each well (Bhatia et al., 2009).

Ethical approval

Ethics committee approval was obtained for the study from Pinnacle Biomedical Research Institute (PBRI) Bhopal Ethics Committee with the date 24.08.2019 and decision number PBRI/IAEC/PN-19016.

RESULT AND DISCUSSION

Development of liposomes

Liposomes prepared with three lipids Phospholipon 90H, Phospholipon 100S, and soya phosphatidylcholine exhibited vesicle size from 182 to 995nm. Liposomes prepared using Phospholipon 100S (182.4±2.4 nm, 0.123) appeared to have a smaller size, size distribution, and PDI when compared with Phospholipon 90H (835.5±5.4nm, 0.519) and soya phosphatidylcholine (779.3±3.7 nm, 0.579), as shown in Table 1.

Liposomes with Phospholipon 100S showed lower PDI values, indicating smaller size distribution compared to others. The small vesicle size of the liposome formulation made up of Phospholipon 100S was shown to have a higher entrapment efficiency value (L11, 89.9% ±1.9).

The liposomes stabilized by using the cholesterol in their structure. Rigidity improved the leakage of drug from bi-lipid layers prevented by the use of cholesterol. It also reduced the flexibility of the liposomes, which increases the stability. The entrapment efficiency improved due to prevention of leakage and flexibility.

The cholesterol-lipid ratio was optimized for 1:1, 1:2, 1:3, and 1:4. It was witnessed that by increasing the amount of cholesterol and lipid the percent entrapment efficiency of the drug was increased gradually. The vesicle size and PDI also increased as the ratio of cholesterol-lipid was increased. In the preliminary studies, it was found that if EE is >85% then there is no effect of sonication time. Hence sonication time was optimized to 15 minutes based on the results of vesicle size, PDI, and EE (%).

A 3^2 full factorial design was employed in the optimization of liposome development. In the used design two factors are evaluated, each at three levels. All nine possible combinations were performed in an experimental trial. The amount of Phospholipon-100S (X_1) and the time for rotation (X_2) were selected as independent variables. The entrapment efficiency (%) and vesicle size (nm) were selected as dependent variables. ANOVA was applied to detect insignificant factors. The fit of the model was dependent upon the lower p-value, high F-value, high level of adjusted R^2, and predicted R^2 (Table 2). From the data of entrapment efficiency (%) of the factorial formulations OL1 to OL9, polynomial equations for entrapment efficiency (%) had been derived using Design Expert 11 software. The co-

efficients for entrapment efficiency (%) (Y1) and Vesicle Size (nm) (Y2) of the factorial formulations are shown in Table 2. In the case of entrapment efficiency (%) (Y1), the positive sign for coefficients of X1 indicate that as the amount of Phospholipon-100S increases, entrapment efficiency (%) increases. The negative sign for coefficients of the time for rotation (X2) indicates that as the time increases, entrapment efficiency (%) decreases. In the case of vesicle size (nm) (Y2), the positive sign for coefficients X1 and X2 indicated that as the concentration of Phospholipon-100S and the time for rotation (X2) increases, Vesicle Size (nm) increases.

The validity of the optimization model was verified using four extra design checkpoint formulations (LC1 to LC4) and determining their entrapment efficiency (%) and vesicle size (nm). The nearness of the predicted and observed values of LC1 to LC4 in the method indicates by their low value of percent residual value. So results also proved the derived equations for the dependent variable for entrapment efficiency (%) and vesicle size (nm) (Table 3) predicted by software. The software-generated contour plots of response surface optimization method for the dependent variable are shown in Figures 1A and 1B, respectively.

The optimized batch (FL1) was prepared for the entrapment efficiency (85%) and vesicle size (625 nm). The software suggested the amount of Phospholipon-100S (3%, w/w) and time of rotation (15.5 minutes) to get optimized results. Here, a very low deviation was observed in predicted value and observed value. The average vesicle size of the optimized batch (FL1)

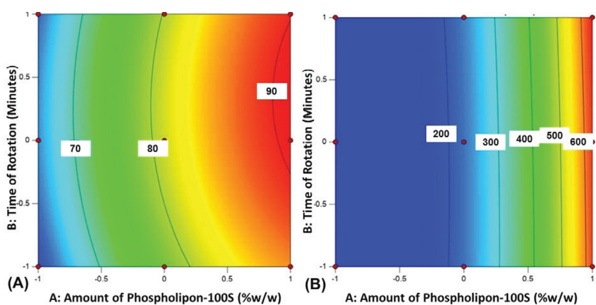

Figure 1. Contour plots indicating the relationship between the factors A and B on the response variables, (A) Entrapment Efficiency (%) (B) Vesicle Size (nm).

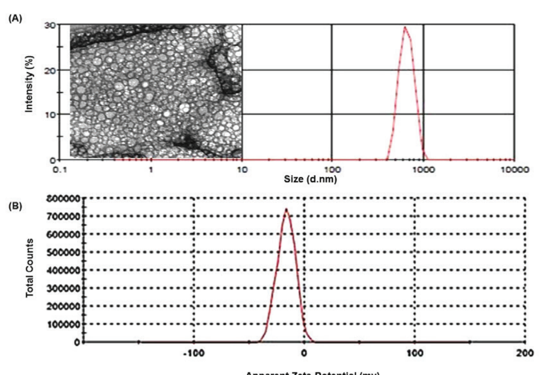

Figure 2. (A) Vesicle size, insert TEM photograph and (B) zeta potential distribution of optimized bexarotene liposome formulation (FL1).

was found to be below 639 nm and PDI was found to be below the desired value of 0.15 (Figure 2A). The zeta potential of the optimized formulation was observed to be -19.3 mV (Figure 2B). TEM results showed the lamellarity and morphology of optimized bexarotene liposomes. (Figure 2A, insert).

Development of bexarotene liposomal topical gel

Several carbopol concentrations (0.5 to 1.75%, w/w) were used and the best was selected based on viscosity, consistency, gel-forming capacity, and spreadability. Except for G1, all other formulations gave sufficient viscosity, consistency, and spreadability to enable topical application that was homogenous and non-greasy in nature and appearance. It was observed that the topical gel dried and made a hard film a while after application. To elude the drying nature of the topical gel, isopropyl alcohol, methyl paraben, and propyl paraben were added to the liposomal topical gel. The values of various evaluation parameters were found for the drug content (93 to 97%), viscosity (1292 to 5620 cps), spreadability (3.8 to 4.4 cm), and pH value (6.7 to 6.9). The high spreadability value shows that the liposomal topical gel was spread easily with a small application of stress. The high value of spreadability was useful in CTCL for even distribution and spreading of liposome on the dorsal skin surface. The rheological behavior of the liposomal topical gel was also responsible for the spreadability and residence time of topical gel on the skin surface. If viscosity values are between 4000 to 5000 cps at 25 ºC with a fixed speed of 12 RPM with spindle S-62, LVDV-E is ideal for topical gel. Rheological behavior was interpreted by the viscosity values optimized against shear rates and from the results it was observed that the viscosity reduced with an increase in shear rate. So, the formulated topical gel was having pseudo-plastic behavior, which was ideal for the topical formulation and application (Saka et al., 2020).

In vitro drug release and *ex vivo* permeation studies

For release studies of bexarotene drug solution and bexarotene liposome formulation, the withdrawn samples from the dialysis apparatus were diluted and analyzed. For the drug solution dialysis, the complete release was perceived within 10 h (Figure 3), whereas from the liposome formulation, drug release was found in a controlled manner and 60% of the drug was released within 12 h. In the study of drug diffusion from liposomal topical gel from Franz

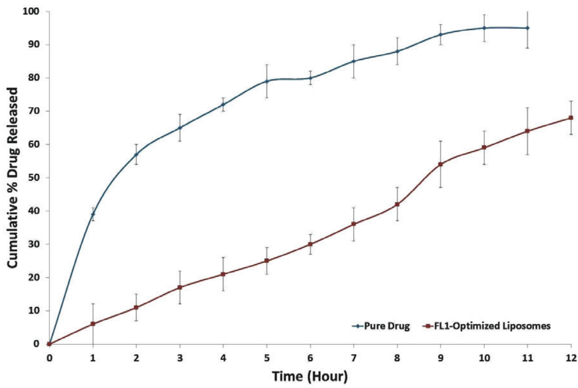

Figure 3. Comparative in-vitro dissolution profile of pure bexarotene drug and optimized liposome formulation (FL1) for each time interval in phosphate buffer (pH 7.4).

diffusion cell, the results depicted that more than 50% of drug was diffused through the cellophane membrane in 8 h. Likewise, in the ex-vivo diffusion model the bexarotene liposomal topical gel gave similar results as in the in-vitro diffusion studies. These outcomes demonstrate that the drug is being released in a sustained and controlled manner over a prolonged period of time, which is important for skin disorders like CTCL where once or twice a day application is sufficient, thus improving patient compliance.

To determine the penetrative property of topical gel formulation, dermal uptake studies were carried out using drug disposition studies. The amount of drug deposited in the skin was also calculated. It was concluded that formulation LG5 showed the highest (31.29%) restoration of the drug in the skin. It showed that the high concentration of drug was available in the skin for a long period of time for local application of the drug in the effective management of CTCL (Figure 4).

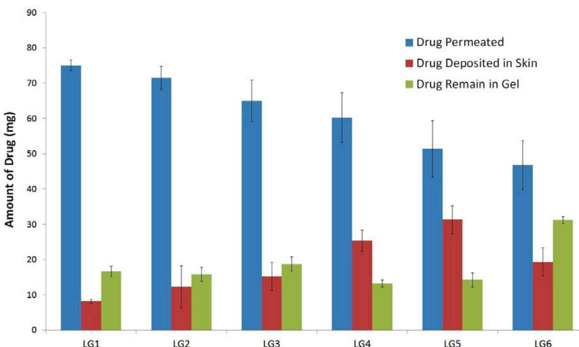

Figure 4. Ex vivo skin permeation study and drug deposition (retention) study of optimized liposomal topical gel formulations following transdermal application.

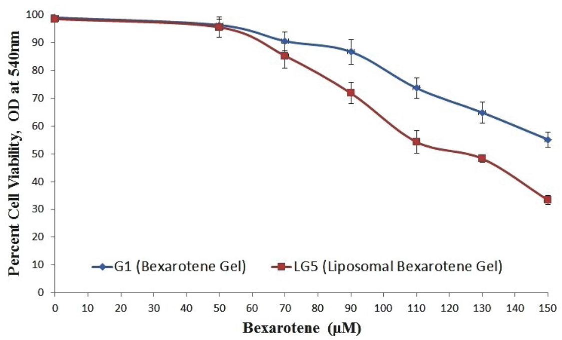

Figure 5. Cytotoxicity activity of pure bexarotene (G1, IC_{50} >150 µM) and optimized liposomal topical gel formulations (LG5, IC_{50} ~128 µM*) in Hut 78 cell line (n=6, StatTM Software, P<0.05;*implies Significant Changes Observed).

Skin-irritation study

The liposomal topical gel (LG5) is topically applied and used with UV radiation for more effective treatment and management of CTCL. The skin irritation studies were observed under UV light exposure. It was found that the liposomal topical gel reduced the wrinkle score of the skin. The results clearly show that the topical gel does not produce any irritation to the skin while in the presence of UV light (Table 5).

In-vitro cell proliferation studies

The cytotoxic activity was performed by determining the minimum inhibition concentration of optimized pure drug-loaded gel (G1) and liposomal topical gel of bexarotene (LG5) formulations using the standard MTT cell viability assay. The results of the MTT cell viability method depicted IC50 and value of > 150 µM and 128 µM by G1 and LG5 formulation against the Hut 78 cell line associated with CTCL, respectively. The result revealed that the amount of drug needed for preventing cell line proliferation is maximum by bexarotene-loaded topical gel than other nano-vesicular formulations (Liposomes) as shown in Figure 5, and the absorbance was measured at 263 nm using a plate reader (BioRad). An ANOVA test was employed to find the statistical significance of the observed value.

CONCLUSION

The liposomal topical gel of bexarotene was designed, formulated, and evaluated for various parameters. The liposomes were prepared by the optimization technique for desired vesicle size, high drug loading, and stable dosage form. The promised liposomes were incorporated into Carbopol topical gel and characterized for different parameters. The prepared topical gel has good spreadability and rheological properties. The promised liposomal topical gel had shown controlled diffusion of drug and sufficient deposition of drug in the skin. This study indicates that the liposome topical gel of bexarotene can be considered in the management of CTCL.

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Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- S.S.,N.S., S.P.V., D.P.B.; Data Acquisition- S.S.,N.S., S.P.V.; Data Analysis/Interpretation- S.S.,N.S., S.P.V., D.P.B.; Drafting Manuscript- S.S.,N.S., S.P.V., D.P.B.; Critical Revision of Manuscript- S.S., D.P.B.; Final Approval and Accountability- S.S.,N.S., S.P.V., D.P.B.

Conflict of Interest: The authors have no conflict of interest to declare.

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Original Article

Formulation, development and evaluation of an osmotic drug delivery system for lornoxicam

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ABSTRACT

Background and Aims: The current study aimed to develop an osmotic drug delivery system for Lornoxicam to prolong its release.

Methods: Two different approaches were used for development of the osmotic drug delivery system; the first one was to formulate a controlled porosity osmotic tablet and the other was to design an elementary osmotic tablet. The controlled porosity osmotic tablets were coated with different concentrations of osmogents, a pore former, and varying coating thicknesses, and the effects were observed on the release. In the elementary osmotic pump, the tablets were drilled to make an orifice that creates pressure to release the drug by osmosis, and drug release was studied.

Results: The formulations were evaluated for different parameters namely appearance, uniformity of weight, drug content, hardness, and drug release. Also, the effect of different osmotic agents responsible for developing the osmotic pressure such as sodium chloride and mannitol along with the different concentrations of pore-forming sorbitol were studied. A comparison was made between the controlled porosity osmotic tablet in which the membrane coating contains water-soluble pore-forming polymers that leach when the membrane comes in contact with water thereby permitting water inside the wall and creating the osmotic pressure to release the drug, and the elementary osmotic tablet containing the osmotic agent sodium chloride coated with the rate-controlling semipermeable membrane, cellulose acetate, which contains an orifice of a critical size through which the drug is delivered.

Conclusion: From the results, it was found that the developed formulation of the controlled porosity osmotic tablet was able to release Lornoxicam (CPOP) over 12 hours at zero-order kinetics and, the concentration of the osmotic agent, level of pore former, and thickness of the membrane coating are responsible for controlling the release of lornoxicam. The membrane coating was subjected to SEM analysis, which showed the formation of pores in the membrane. The developed controlled porosity osmotic pump tablet of lornoxicam was found to control the drug release for 12 hours.

Keywords: Lornoxicam, Controlled porosity osmotic tablet, elementary osmotic tablet, drug release, osmotic agent, pore former & scanning electron microscopy

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INTRODUCTION

Oral administration is one of the ancient and widely used routes for effective and convenient drug delivery with both local and systemic effects (Theeuwes, 1975). Conventional preparations are usually administered twice or three times a day, which leads to a large fluctuation in drug plasma concentration and side effects on the human body. Constant plasma levels can offer a therapeutic advantage for many drugs in terms of both efficacy and tolerance of the treatment (Theeuwes, 1975). Once-daily controlled-release formulations are often desirable. Osmotic drug delivery is a reliable and convenient controlled drug delivery system (CDDS). Osmotic pressure acts as a driving force for these systems to release the drug in a controlled manner. The osmotic pump tablet (OPT) generally consists of a core including the drug(s), an osmotic agent, other excipients, and a semipermeable membrane coat. Osmosis is a phenomenon that tends to achieve a zero-order drug release. It acts as a driving force for the release of drugs from the dosage form. The osmotic tablet works on the principle of osmosis i.e., water moving through a permeable membrane driven through the difference in osmotic pressure across the membrane. It is driven by a difference in solute concentration across the membrane that allows the passage of water but rejects most solute molecules or ions. Based on this principle, osmotic drug delivery results in better drug release independent of the concentration of the drug. The aim of controlled release drug delivery is to sustain the action of the drug at a determined rate by maintaining a constant and effective drug level in the body with minimum side effects (Zenter, McClelland, & Sutton, 1991; Santus & Baker, 1995; Herbig, Cardinal, Korsmeyer, & Smith, 1995; Patel et al., 2010).

The osmotic pump tablet is preferred among the controlled release systems and has many advantages, such as reducing the risk of adverse reaction, improving the compliance of patients, and exhibiting comparable *in vitro/in vivo* drug release. Drug substances can be delivered in a controlled pattern over a long period by osmotic pressure with the increased interest in the development of osmotic devices over the past two decades. Delivery of drug substances from osmotic systems is not affected by the varying physiological factors within the gut lumen and the drug release patterns can be easily predicted from the known properties of the drug substance and the dosage form (Pritam, Braj, Ambikanadan, Prakash, & Rajesh, 2007; Kumar, Singh, & Mishra, 2009; Syed, Farooqui, Mohammed, Dureshahwar, & Farooqui, 2015). Lornoxicam, from the class of oxicam cluster of non-steroidal anti-inflammatory drugs, is used as an analgesic and anti-inflammatory. Its commercially available dosage forms includes conventional immediate-release tablets 4 mg/8 mg, rapid release 8 mg tablets, and parenteral formulations of 4 mg/ ml for intravenous and intramuscular use. Lornoxicam has been widely used for the treatment of pain and inflammation in patients with osteoarthritis and rheumatoid arthritis, pre-operative and post-operative pain associated with gynecological, orthopedic, abdominal, and dental surgeries. As lornoxicam has a half-life of 3-5 hours, a dosing frequency of twice or thrice a day, and intermediate solubility in water, it was selected for the development of osmotic drug delivery system dosage forms (Santus et al., 1995; Govt. of India 1996; Syed, et al., 2015).

In 1974, Theeuwes invented an elementary osmotic pump (Theeuwes, 1975). The elementary osmotic pump delivers the drug by the osmotic process at a controlled rate. The control rests in: *a)* water permeation characteristics of a semi-permeable membrane surrounding the formulating agent *b)* osmotic properties of the formulation. This system contains an osmotically active agent surrounded by the rate-controlling semi-permeable membrane. The device is created by a drug having appropriate osmotic pressure into a tablet using a tableting machine followed by coating the tablet with a semi-permeable membrane and drilling of small hole through the membrane (size varies from 0.5 to 1.5 mm).The drilling may be done by mechanical drilling or laser drilling (CO_2 laser beam with a wavelength of 10.6μ) (Herbig et al., 1995; Patel et al., 2010). On exposure of the dosage form to the aqueous environment, the imbibition of water occurs by the core osmotically at a controlled rate and is determined by the permeability of the membrane and osmotic pressure of the core formulation. The volume of saturated drug solution delivered is equal to the volume of solvent uptake (Chai, Xu, & Liu, 2008). The advantages of this system include being easy to develop, suitable for the drug having moderate solubility, and economical. The disadvantages are that the size of the hole is critical, and blockage of the orifice is possible (Pritam et al., 2007; Chai et al., 2008; Kumar et al., 2009; Edavalath, Shivanand, Kalyani, Prakash, & Goli, 2011 Syed, et al., 2015).

For design of controlled porosity osmotic pump; the pump can be made with a single or multi-compartment dosage form. In either form, the delivery system comprises a core with the drug surrounded by a membrane that has an asymmetric structure, i.e., it comprises a skinny layer with a supporting porous substructure. The membrane is formed by a phase inversion process in which controlled by the evaporation of a mixed solvent system. The membrane is porous to water, however, rubberizes to solutes and is insensitive to pore-forming additives dispersed throughout the wall. When exposed to water, low levels of water-soluble additives are leached from the polymer materials that were permeable to water yet remained insoluble. The resulting sponge-like structure forms the controlled porosity walls of interest and is substantially permeable to both water and the dissolved drug agents (Liu et al., 2007; Chai et al., 2008; Kumar et al., 2009; Edavalath et al., 2011; Syed et al., 2015).

The current study aimed to develop an osmotic drug delivery system for Lornoxicam. Two different approaches were used for the formulation. The first one was to formulate a controlled porosity osmotic tablet and the other was to design an elementary osmotic tablet. The formulations were evaluated for different parameters namely appearance, uniformity of weight, drug content, hardness, and drug release pattern. In addition, the effect of different osmotic agents responsible for developing the osmotic pressure such as sodium chloride and mannitol along with the different concentrations of pore-forming sorbitol were studied. The controlled porosity osmotic tablet

in which the membrane coating contains water-soluble pore-forming polymers that leach when the membrane comes in contact with water thereby permitting water inside the wall, and creating the osmotic pressure to release the drug were compared with the elementary osmotic tablet containing the osmotic agent coated with the rate-controlling semi-permeable membrane which contains an orifice of a critical size through which the drug is delivered.

MATERIALS AND METHODS

Materials

Lornoxicam was obtained as a gift sample from Piramal Healthcare, Mumbai, India. Sodium chloride, and mannitol were obtained from Universal Laboratories Pvt. Ltd, Mumbai, India. Cellulose acetate was obtained from Shreya Life Sciences, Aurangabad. PVP k-30 and Sodium Lauryl sulfate were obtained from Merck Specialities Pvt. Ltd, Mumbai, India.

Analytical method

Preparation of standard stock solution: The standard drug solution of lornoxicam was prepared by dissolving 10 mg in phosphate buffer pH 7.2 and the volume was made up to 100 ml to obtain a stock solution concentration of 100 µg/ml. Ultrasonication procedure was applied to obtain a clear solution (Liu et al., 2007; Edavalath et al., 2011; Syed, & Mohammed, 2014)

Determination of measurement wavelength: From the standard stock solution, 1 ml was pipetted out into a 10 ml volumetric flask. The volume was made up to 10 ml with a phosphate buffer solution of pH 7.2. The resulting solution containing 10 µg/ml was scanned between 200 and 400 nm. (Liu et al., 2007; Edavalath et al., 2011; Syed, & Mohammed 2014).

Preparation of calibration curve of Lornoxicam in phosphate buffer pH 7.2: Aliquots of 0.2 to 1.4 ml portions of stock solutions were transferred to a series of 10 ml volumetric flasks, and the volume was made up to the mark with phosphate buffer pH 7.2. Serial dilutions of the range of 2, 4, 6, 8, 10, 12, and 14 µg/ml were prepared. The absorbance was measured at λ_{max} 376 nm (Liuet al., 2007; Edavalathet al., 2011; Syed**,** & Mohammed, 2014).

IR spectroscopic analysis: The identification of Lornoxicam was done by FTIR spectroscopy. The IR absorbance spectrum of Lornoxicam was recorded using a Jasco-4100 spectrometer over a range of 400 to 4000 cm^{-1} at a resolution of 2 cm^{-1}. KBr powder was dried at 60 °C for one hour. The dried KBr powder was mixed uniformly with the drug and IR spectra were taken for this mixture (Liuet al., 2007; Edavalathet al., 2011; Syed**,** & Mohammed, 2014).

Solubility study

The solubility of the compound was carried out in water, 0.1 N HCl, and phosphate buffer pH 6.8, pH 7, pH 7.2, and pH 7.4. An excess amount of drug was dissolved in 5 ml of solvent. The solution was then subjected to ultrasonication for 30 minutes. It was then allowed to stand for 24 hours at room temperature in tightly closed vials to attain a saturation equilibrium. After 24 hours, the solution was filtered through Whatman filter paper No 41. It was then diluted appropriately with the solvent and was analyzed at 376 nm by UV Spectrophotometry (Liuet al., 2007; Edavalathet al., 2011; Syed**,** & Mohammed, 2014).

Excipients compatibility study

It is very important to perform a physicochemical evaluation of all excipients which are probably used in the formulations. While most excipients have no direct pharmacological action, they do perform either useful tasks or damaging actions (such as speeding up the degradation of the drug). Interactions in the solid state between the active ingredient and the excipients in pharmaceutical dosage forms can give rise to changes in the stability, solubility, dissolution rate and bioavailability of drugs. The IR study was performed between drug and the excipients (Liuet al., 2007; Edavalathet al., 2011; Syed**,** & Mohammed, 2014).

Preparation of osmotic core tablet

The osmotic core tablets were prepared by the wet granulation method. The granules were prepared by the non- aqueous (IPA) granulation technique. Lornoxicam and all the excipients previously passed through ano. #60sieve. Then, Lornoxicam was mixed with all the excipients except the binding and solubilizing agents as per the formulas given in Table 1-2 The blend was mixed for 10 minutes in a polybag and later, the mixture was granulated with a PVP K-30 (binder) in isopropyl alcohol (IPA) (solvent for wet granulation) and wetting/solubilizing agents. The resulting wet mass was passed through a no. #25 sieve and the granules were dried at 50 °C for 15 minutes to obtain a loss on drying (LOD) value between 1% and 1.2%, after which they were passed through a no #30 sieve and compressed using tablet machine (Rimek mini press-II, Gujrat, India) .

The core tablets containing 8 mg of lornoxicam for primary batches were formulated using NaCl (16, 24, 32 mg) as an osmogent in 1:2, 1:3, 1:4 proportions respectively with a drug to study the consistency of the coating (an excess amount of osmogent may cause bursting of the external coat or membrane) (Table 3). Sodium lauryl sulfate (SLS) was added as a solubilizer (5%) to increase the dissolution of Lornoxicam. Lactose and microcrystalline cellulose were used as diluents to adjust the bulk, magnesium stearate was added as a lubricant to prevent sticking, and talc was used as a glidant to promote the uniform flow of powder (Table 4). The core tablets were coated with a coating material containing 4% w/v of cellulose acetate (4 g/100 ml) with 22% and 30% w/w sorbitol equivalent to the weight of cellulose acetates solution (Table 5), such as 0.88 g (22%) and 1.2 g (30%) as the pore former or channeling agent in the solvent system which is the mixture of acetone and isopropyl alcohol (IPA) in the ratio 90:10 (90 ml acetone and 10 ml isopropyl alcohol, two solvents were mixed acetone and isopropyl alcohol) and maintained the weight gain of 3%, 4% and 5% of the tablet for each formulation, respectively (Table 1), which contain the coating weight gain of formulation.

In another formulation, mannitol was used instead of sodium chloride as the osmotic agent in the proportion of 1:5, 1:10, and 1:20 with the drug (Table 6). Excipients, like solubility modifiers, diluents, and lubricants were added and coated with 4% w/w cellulose acetate and 22% w/w pore former (sorbitol) and maintained the weight gain of 3%, 4%, and 5% of a tablet for each formulation respectively, as provided in Table 1-2.

Elementary osmotic pump (EOP) tablets of Lornoxicam were prepared which contained the osmogent (NaCl) in the ratio 1:2 (16 mg), 1:3 (24 mg), and 1:4 (32 mg) with 4% weight gain without pore-forming agent (sorbitol). The elementary osmotic tablets were drilled with a mechanical driller with manual rotation (PCP driller) of orifice size 0.8 mm. Figure 4 represents the mechanical driller for the orifice in the EOP tablet and coated with 4% w/w cellulose acetate without pore former. The coating weight gain was maintained at 4% for each tablet, as per Table 1-2.

Table 1. Primary Batches of an osmotic tablet with sodium chloride.

Ingredients (mg)	LOX01	LOX02	LOX03	LOX04	LOX05	LOX06	LOX07	LOX08	LOX09	LOX10	LOX11	LOX12	LOX13	LOX14	LOX15
Core Tablet Lornoxicam	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
Sod. Chloride	--	16	24	32	16	24	32	16	24	32	16	24	32	16	24
MCC	162	146	138	130	146	138	130	146	138	130	146	138	130	146	138
Lactose	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
SLS	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
PVP K-30	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
IPA	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Mag. Stearate	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Talc	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Coating Wt gain %	3%	3%	3%	3%	4%	4%	4%	5%	5%	5%	3%	3%	3%	4%	4%
Pore former (Sorbitol%)	22%	22%	22%	22%	22%	22%	22%	22%	22%	22%	30%	30%	30%	30%	30%

Table 2. Primary Batches of an osmotic tablet with sodium chloride/mannitol.

Ingredients (mg)	LOX16	LOX17	LOX18	LOX19	LOX20	LOX21	LOX22	LOX23	LOX24	LOX25	LOX26	LOX27	LOX28
Core Tablet Lornoxicam	8	8	8	8	8	8	8	8	8	8	8	8	8
Sod. Chloride/ Mannitol LOX20 onwards	--	16	24	32	16	24	32	16	24	32	16	24	32
MCC	162	146	138	130	146	138	130	146	138	130	146	138	130
Lactose	50	50	50	50	50	50	50	50	50	50	50	50	50
SLS	12	12	12	12	12	12	12	12	12	12	12	12	12
PVP K-30	12	12	12	12	12	12	12	12	12	12	12	12	12
IPA	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.	q.s.
Mag. Stearate	3	3	3	3	3	3	3	3	3	3	3	3	3
Talc	3	3	3	3	3	3	3	3	3	3	3	3	3
Coating Wt gain %	3%	3%	3%	3%	4%	4%	4%	5%	5%	5%	3%	3%	3%
Pore former (Sorbitol%)	22%	22%	22%	22%	22%	22%	22%	22%	22%	22%	30%	30%	30%

Table 3. Primary batches of EOP osmotic tablet (with Sodium chloride).

Ingredient (mg)	EOP 01	EOP 02	EOP 03	EOP 04
Core tablet				
Lornoxicam	8	8	8	8
Sodium chloride	--	16	24	32
Microcrystalline cellulose	162	146	138	130
Lactose	50	50	50	50
SLS	12	12	12	12
PVPK-30	12	12	12	12
IPA	q.s	q.s	q.s	q.s
Magnesium stearate	3	3	3	3
Talc	3	3	3	3
Coating ingredient				
Wt gain (%)	4%	4%	4%	4%

Table 4. Formulations of core tablets with Sodium chloride (Osmogent).

Ingredients (mg)	LOX A0	LOX A	LOX B	LOX C
Lornoxicam	8	8	8	8
Sodium chloride	--	16	24	32
Microcrystalline cellulose	162	146	138	130
Lactose	50	50	50	50
SLS	12	12	12	12
PVPK-30	12	12	12	12
IPA	q.s	q.s	q.s	q.s
Magnesium stearate	3	3	3	3
Talc	3	3	3	3
Tablet weight	250	250	250	250

Table 5. Formulation of the coating solution for lornoxicam core tablets.

Ingredients	F1	F2
Cellulose acetate	4 % w/v	4 % w/v
PEG 400	12.5 % w/w	12.5 % w/w
Sorbitol	22 % w/w	30 % w/w
Acetone:IPA	90:10 v/v	90:10 v/v

Table 6. Formulations of core tablets with Mannitol (Osmogent).

Ingredients (mg)	LOX D	LOX E	LOX F
Lornoxicam	8	8	8
Mannitol	40	80	160
Microcrystalline cellulose	132	92	52
Lactose	40	40	40
SLS	12	12	12
PVPK-30	12	12	12
IPA	q.s	q.s	q.s
Magnesium stearate	3	3	3
Talc	3	3	3
Tablet weight	250	250	250

The polymers and their concentrations were chosen based on the literature as well as results obtained from primary batches.

Evaluation of uncoated osmotic tablets

Measurement of thickness and diameter

The uniformity of the diameter and thickness was measured using a vernier caliper. The average diameter and thickness of the tablet were calculated. The test passed if none of the individual diameter and thickness values deviated by ± 5% of the average.

Hardness

The Monsanto hardness tester model VMT-239 manual (VinSyst) manufactured by VinSyst technologies Mumbai was used to check the hardness of the tablet. The tablet was placed vertically between the jaws of the tester. The two jaws are placed under tension by a spring and screw gauge. By turning the screw, the load was increased and at collapse, the applied

pressure from the spring was measured in kg/cm^2. The mean ± SD was calculated.

Friability

Twenty tablets, as prescribed in the Indian Pharmacopoeia, were weighed and placed in a Roche friabilator (Electrolab, India). Twenty reweighed tablets were rotated at 25 rpm for 4 min. The tablets were then dedusted and re-weighed and the percentage of weight loss was calculated. The percentage friability of the tablets was measured as per the following formula,

$$\% \text{ loss} = \frac{\textit{Initial wt. of tablets - Final wt. of tablets}}{\textit{Initial wt. of tablets}} \times 100$$

Uniformity of dosage form

To study weight variation, 20 tablets were weighed individually using an electronic balance and the test was performed according to the official method. The average weight was calculated from the total weight of the 20 tablets. The individual weights were compared with the average weight. Since the average weight of the tablet was250 mg, the percentage difference in the weight variation should be within the permissible limits according to the Indian Pharmacopoeia; the limit for weight variation for tablets weighing 250 mg or more is ± 5 %. The test requirements are met if not more than 2 tablets of individual weight deviate from the percentage, i.e., 5%.

Drug content uniformity

Ten tablets were weighed and the average weight was calculated. All tablets were crushed, powder equivalent to 8 mg drug was dissolved in 8 ml of 0.1 N NaOH and the volume was made up to 100 ml with pH 7.2 phosphate buffer. The solution was shaken for 1 hour and kept at room temperature for 24 hour. From the stock solution, a 1 ml aliquot was transferred to a 10 ml empty volumetric flask and the volume was made with pH 7.2 phosphate buffer. The solution was filtered and the absorbance was measured spectrophotometrically at 376 nm against a pH 7.2 phosphate buffer as a blank. The amount of drugs contained in one tablet was calculated.

Preparation of coating solution

The coating solution containing cellulose acetate and sorbitol (pore-forming agent) was prepared as per the formula given in Table 5. An accurately weighed quantity of cellulose acetate was added to acetone (90%). The mixture was stirred until a clear solution was formed. The weighed quantity of sorbitol was dissolved in 2 to 4 ml of distilled water, then this solution was added to IPA (10%) and the solution was added slowly to the cellulose acetate solution. The mixture was stirred continuously for 30 minutes. Then the solution was filtered through a muslin cloth.

Coating of lornoxicam osmotic core tablet

The solution of cellulose acetate in acetone: IPA (90:10, v/v) was used to achieve a weight gain of approximately 3-5 % per tablet. The core tablets were film-coated in a conventional pharma R & D coater (mfg by- Ideal cures Pvt. Ltd India), 4 inches with 3 baffled stainless-steel pans by a spray coating process. The coating parameters were optimized on placebo tablets made of lactose monohydrates and 0.5% magnesium stearate. Initially, the tablets were kept at 40 °C for 10 minutes while the pan rotated at 15 rpm.

The rotating speed was then increased to 15 to 30 rpm and the coating solution was sprayed at a rate of approximately 1-2 ml/min. The atomizing pressure was adjusted to 1-2 kg/cm^2, and the inlet and outlet temperatures were varied from 35-55 °C. The process was continued until the whole solution was sprayed onto the tablets. The coated tablets were rotated for a further 15 min under the blower. The coating process parameters were optimized concerning coating pan speed, coating pan inlet air temperature, atomizing air pressure, and spray rate.

Evaluation of lornoxicam osmotic coated tablet

Percentage weight gain

From the batch of lornoxicam tablets, 30 core tablets were randomly selected and subjected to coating. The initial weight of 30 uncoated tablets was recorded. After a period of coating, the spraying of the coating solution was stopped and the tablets were allowed to dry for 10–15 min in the coating pan at 45 °C to remove the majority of the solvent moisture. The weight of the 30 coated tablets was recorded. The percent weight gain was calculated.

The thickness of film

Three tablets of each batch were evaluated for the thickness of the film. After dissolution, the tablet shell was cut with help of a cutter and washed with water to obtain a clear film. The thickness of the film was measured using a screw gauge.

Scanning electron microscopy (SEM)

Coating membranes of formulation obtained before and after complete dissolution of core contents were examined for their porous morphology by scanning electron microscope (SEM). Before dissolution, the tablets were cut with a sharp blade, and the coating membrane was taken out. This membrane was cleaned with a dried cloth to remove any adherent particles and was used for SEM. Similarly, the coating membrane was taken out from the tablets after 12 hr. of dissolution study and was used for SEM. The coating membrane was carefully washed 3 to 4 times with water to remove any adherent solid particles. Coating membranes were dried at 45 °C for 12 hours and stored between sheets of wax paper in a desiccator until examination. The small pieces of coating membranes were placed on a spherical brass stub (12 mm diameter) with a double-backed adhesive tape in such a way that the outer portion of the coating membrane was in front of an electronic beam and was examined under a scanning electron microscope.

Dissolution studies

The release rate of Lornoxicam from CPOP and EOP (n=3) was determined from all the batches. Batches were evaluated by studying the release for the first 2 hours in 900 ml dissolution medium of 0.1 N HCl, then the remaining 10 hours in 900 ml dissolution medium of phosphate buffer pH 7.2 using a USP type II (Paddle) dissolution apparatus with 100 rpm at 37±0.5 °C. The samples (5 ml) were withdrawn at an interval of 1, 2, 3, 4, 6, 8, and 12 hours with the addition of fresh buffer solution (5 ml) maintaining the sink condition. The withdrawn samples

were replaced with a fresh dissolution medium. The samples were filtered through Whatman filter paper and analyzed spectrophotometrically at 376 nm.

Drug release kinetics

The dissolution profile of all the batches was fitted to zero-order kinetics, first-order kinetics, Higuchi, Hixson-Crowell, and Korsmeyer-Peppas models to ascertain the kinetic modeling of drug release by using a PCP Disso Version 2.08 software. The model with the highest correlation coefficient was considered to be the best model. To know the drug release mechanism, the data was further analyzed by the Korsmeyer-Peppas equation and the value of n, i.e., the release exponent, was calculated. The drug release profiles for CPOP and EOP were compared.

Statistical analysis

The release rate of different formulations was compared using one-way ANOVA at $p<0.5$. The statistical analysis was performed using Graph Pad InStat version 3.10.

RESULTS

Solubility study

The solubility of Lornoxicam was determined in various mediums. Thus, from the results, the drug shows pH-dependent solubility. The solubility data is given in Table 7.

Compatibility of the drug with excipients

To check the interaction between the drug and polymers used in the formulations, IR studies were performed. In the IR study, it was found that all the prominent peaks which were present in the individual graphs of lornoxicam and polymers were also present in the IR of the physical mixture of the drug and polymers. These peaks were not affected and were prominently observed in FT-IR spectra (Figures 1, 2, and 3).Thus, we can say that no significant interaction between the drug and polymers were observed (Table 8).

Table 7. Solubility study of Lornoxicam.

Sr. no.	Medium	Solubility (mg/ml)
1.	Water	0.257
2.	0.1 N HCl	0.464
3.	Phosphate buffer pH 4	0.396
4.	Phosphate buffer pH 6.8	0.621
5.	Phosphate buffer pH 7.2	1.862
6.	Phosphate buffer pH 7.4	4.120

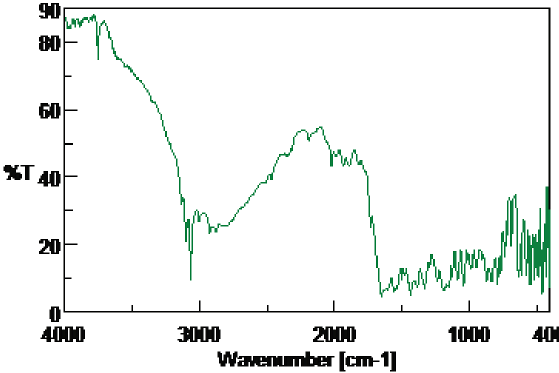

Figure 1. IR Spectrum of Lornoxicam).

Evaluation of core osmotic pump tablet

All formulated osmotic core tablet batches were shiny yellow with a smooth surface, circular curved face and with good texture. The thickness of the tablet was found to be 4.3 to 4.5mm, due to the constant tablet press setting across all batches irrespective of weight variation. Thickness depended on punch size (8.5mm) and tablet weight (250mg); the coefficient of variation (based on 20 tablets/ batch) for each batch was less than ± 5 %, which indicates good thickness uniformity. The diameter of the core of the tablet was 8.5 mm for each formulation. The hardness of the tablet was found to be in the range of 4.0 to 5.2 kg/cm^2. This ensured good mechanical strength. Drug content was uniform within each batch and ranged from 85-115 % of the theoretical value as per Table 9.

Evaluation of coated osmotic pump tablet

Percentage weight gain

To study the effect of weight gain of the coating on drug release, the core tablets of Lornoxicam were coated to obtain tablets with different weight gains (3%, 4%, and 5% w/w) for the entire primary batches of the individual tablet.

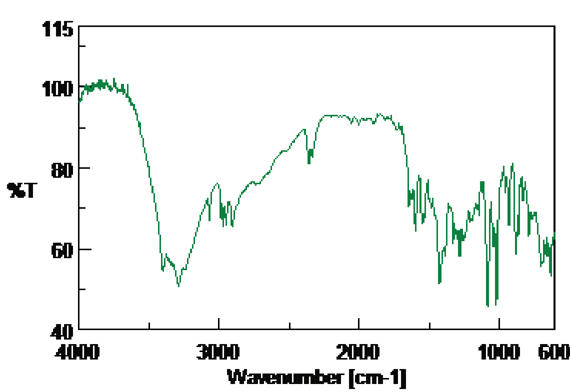

Figure 2. IR of Spectrum of mixture of Lornoxicam and Sodium chloride.

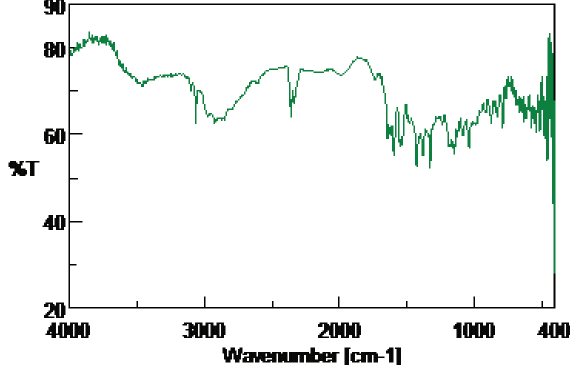

Figure 3. IR Spectrum of mixture of Lornoxicam and Mannitol.

Table 8. IR Study of Lornoxicam with excipients.

Groups	Range	Lornoxicam	Lornoxicam +Sodium chloride	Lornoxicam +Mannitol
-NH (Stretch)	3000-3500	3101.94	3100.19	3100.17
-CONH amide	1630-1680	1651.73	1645.95	1645.95
-NH (bend) sec amide	1550-1640	1555.31	1550.49	1549.52
S=O	1050	1043.3	1146.47	1043.89
C-Cl	540-785	766.56	765.601	768.707
C-H (bend) aromatic ring	690-900	831.16	831.169	830.205

Table 9. Evaluation of Lornoxicam core tablet.

Batch Code	Thickness, Diameter	Friability (%)	Hardness (Kg/ cm²)	Uniformity of weight (mg)	Drug content
LOX A	4.48± 0.01 mm thickness,8.5 mm diameter	0.43 ± 0.03	4.2 ± 0.57	245 ± 2.09	99.01±1.10
LOX B	4.51 ± 0.04 mm thickness, 8.5 mm diameter,	0.55 ± 0.02	5.1 ± 0.76	248 ± 1.48	101.25±1.12
LOX C	4.48 ± 0.03 mm thickness,8.5 mm diameter	0.27 ± 0.01	4.6 ± 0.88	253 ± 2.47	101.91±0.64
LOX D	4.47 ± 0.05 mm thickness, 8.5 mm diameter,	0.74 ± 0.04	4.9 ± 0.54	251 ± 1.98	103.23±1.08
LOX E	4.47 ± 0.08 mm thickness, 8.5 mm diameter	0.65 ± 0.02	4.8 ± 0.58	247 ± 2.86	99.45±2.12
LOX F	4.50 ± 0.02 mm thickness, 8.5 mm diameter	0.13 ± 0.50	5.3 ± 0.43	258 ± 2.06	102.78±1.54

The thickness of the tablet

The thickness of each primary batch was found to be in the range of 4.46 to 4.59 mm.

The thickness of the film

The thickness of the coating film of the primary batches was measured with electronic digital calipers and the mean thickness was calculated. It was 0.067-0.094 mm for each tablet as provided in Table 9.

Hardness

The hardness of the coated osmotic tablets of the primary batches LOX01 to LOX28 was maintained between4.6 -5.8 kg/ cm^2 as provided in Table 9.

Uniformity of weight

The uniformity weight for all batches was found to be between 254 -267 mg as provided in Table 9.

Scanning electron microscopy (SEM)

Cellulose acetate (CA) membranes of the primary formulation of coating solution (F1), obtained before and after dissolution, were studied by SEM. Membranes obtained before dissolution clearly showed a nonporous region (Figure 2). After 12 hours of dissolution, the exhausted membrane containing a plasticizer (PEG 400, 12.5 %) and a pore former (sorbitol, 22 %) clearly showed a microporous region (pores) in the range of 1 to 15 µm (Figure 5). Because sorbitol is present in the coating membrane, the leaching of it from the membrane leads to the formation of pores, and thus the release of the drug takes place.

Figure 4. Mechanical driller for drill the orifice in the EOP Tablet.

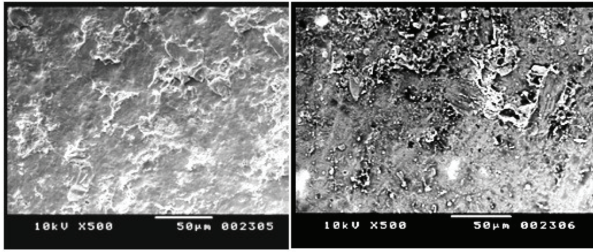

Figure 5. SEM micrograph of coating membranes of primary formulation, before and after dissolution.

Dissolution study

Osmotic tablets were subjected to *invitro* drug release studies in simulated gastric and intestinal fluid. A dissolution study was performed in 0.1 N HCl for the first 2hr and the remaining 10 hr in Phosphate buffer pH 7.2. The results are summarized in Figures 6-8.

Hence, it was evident that when an increase in the concentration of osmogent occurred, the drug release from the system was found to be increased but again reduced the drug release after an increase in the external coat thickness (wt. gain) occurred. The Pore former (sorbitol) produced a significant effect on the release profile. A decrease in the Pore former concentration system failed to release 100% of the drug.

Two types of osmogent (sodium chloride and mannitol) were used in the formulation of CPOP tablets. Both osmogents have different osmotic pressure and to study the effect of the osmogent ratio, core formulations were prepared. The ratios of drug to osmogent, i.e., sodium chloride were 1:2, 1:3, and 1:5 and with other osmogent, i.e., mannitol were 1:5,1:10, and 1:20. All the core formulations were coated with a coating composition, F1 and F2 containing 22% and 30% wt/wt of sorbitol respectively. The release profile from these formulations is shown in Figures 6-8.

DISCUSSION

The results revealed that the formulation LOX01 was devoid of any osmogent in the core and showed less drug release in 12 hours. In the formulation containing a greater amount of osmogent, i.e., sodium chloride, with 22% pore former (sorbitol), LOX04 showed an increase in drug release with 3% weight

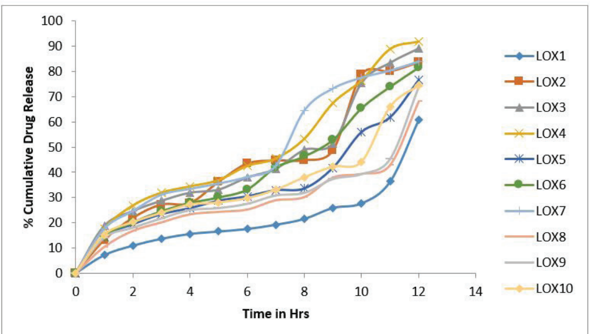

Figure 6. Comparison of *in vitro* release of batches LOX01 to LOX10.

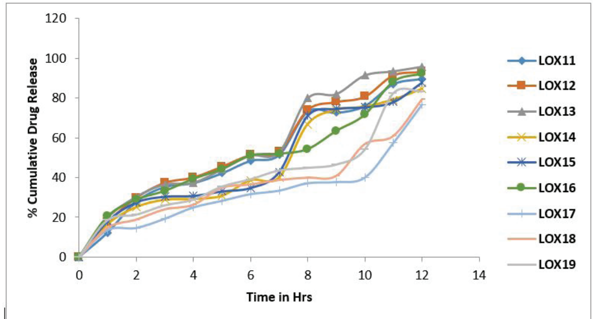

Figure 7. Comparison of *in vitro* release of batches LOX11 to LOX19.

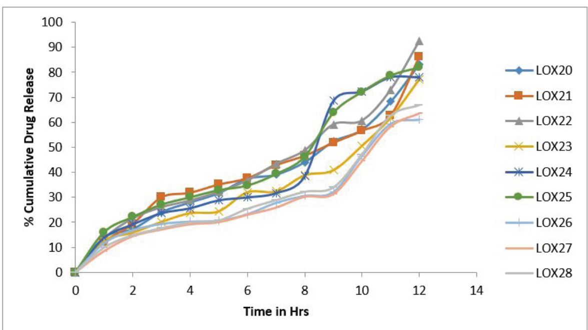

Figure 8. Comparison of *in vitro* release of batches LOX20 to LOX28.

gain compared to 4% and 5% weight gain in 12 hours. In the formulation containing sodium chloride with 30% pore former (sorbitol), LOX13 showed an increase in drug release with 3% weight gain compared to 4% and 5% weight gainin 12 hours. In another formulation set containing mannitol as the osmogent with 22% pore former (sorbitol), LOX22 showed an increase in drug release with 3% weight gain compared to 4% and 5% weight gain in 12 hours.

Effect of drug to osmogent (osmotic agents) ratio

The comparative dissolution profile of all the formulations containing different ratios of drug to osmogent, i.e., sodium chloride in the ratio of 1:4 and mannitol in the ratio of 1:20, gave a better release of the drug from the osmotic tablet. From these release profiles, itis clear that an increase in the concentration of the osmotic agent, the greater the driving force, and this enhanced the release of the drug and thus had a direct effect on drug release. When the coated tablet was exposed to an aqueous environment, water diffused through the film coating (due to the active gradient of water), hydrating the core. The solvation of the osmotic agents created an osmotic pressure difference between the core contents and the external environment, which resulted in greater Lornoxicam release. The CPOP formulation containing sodium chloride as an osmogent showed a better release profile as compared to mannitol because sodium chloride has a higher osmotic pressure than mannitol.

Effect of pore forming level

To study the effect of the pore-forming agents, core formulations of lornoxicam were coated with varying coating compositions of sorbitol as a pore former. Sorbitol was added at 22% and 30% w/w of the coating polymer. The release profile from these formulations is shown in Figures 6-8. The release profile showed that the formulation LOX13 containing 30% w/w of sorbitol released 95.81% of the drug whereas the formulation LOX04 containing 20% w/w of pore former released only 91.82% of the drug as shown in Figure 6-7. The level of sorbitol had a direct effect on drug release. As the level of pore former increased, the membrane became more porous after coming into contact with the aqueous environment, resulting in faster drug release. The level of pore former also affects the burst strength of exhausted shells. The burst strength was inversely related to the initial level of pore former in the membrane. With the increase in the level of sorbitol, the membrane became more porous after exposure to water, leading to a decrease in its strength.

Effect of weight gain

To study the effect of weight gain of the coating on drug release, core tablets of Lornoxicam were coated to obtain tablets with different weight gains (3%, 4%, and 5% wt/wt). The release profile of Lornoxicam from these formulations is shown in comparison for LOX13 and LOX19. The release profile showed that formulations LOX04, LOX13, and LOX22 with 3% weight gain increased drug release more than other formulations, which were coated with 4% and 5% weight gain. Drug release from the 5% weight gain showed much less drug release than 3% and 4% because the cellulose acetate film thickness inhibited the release rate of the drug. Drug release decreased with an increase in the weight gain of the membrane.

Drug release kinetics

The majority of the formulations showed the diffusional exponent, "n", in between 0.5 and 1.0 which indicates the anomalous transport or kinetics which means the drug is released by the combined mechanism of pure non-Fickian diffusion-controlled and swelling-controlled drug release. For some formulations, the "n" value was approximately 0.5 which indicated that the drug was released by a pure diffusion-controlled mechanism (Fickian diffusion). The n ($0.5<n<1$) value also revealed the drug release mechanism via diffusion coupled with erosion. Fickian diffusional release occurred by the usual molecular diffusion of the drug due to a chemical potential gradient and is provided in Table 10.

Comparative study of CPOP and EOP

EOP Lornoxicam tablet was drilled with a mechanical driller having an orifice size of 0.8 mm and delivered the drug up to 12 hours. It was observed that with an orifice size of 0.8mm Lornoxicam released 84.5% of the drug within 12 hours. Based on this release, it was observed that formulations containing no osmogent showed a decreased release of the drug than formulations containing an osmogent. Amongst all the EOP formulations,

Table 10. Drug release kinetics of the formulated batches.

Batch code	R^2					n	k
	Zero-order	1st order	Matrix	Peppas	Hixson Crowell		
LOX01	0.8905	0.8153	0.8069	**0.9216**	0.8436	0.6827	6.2915
LOX02	**0.9731**	0.8997	0.9099	0.9660	0.9305	0.7142	12.1489
LOX03	**0.9570**	0.8755	0.9053	0.9319	0.9171	0.6070	15.3291
LOX04	**0.9722**	0.8894	0.9285	0.9596	0.9365	0.9365	15.6322
LOX05	**0.9463**	0.8889	0.8961	0.9324	0.9174	0.6006	12.2260
LOX06	**0.9799**	0.9233	0.9195	0.9631	0.9525	0.6853	12.0217
LOX07	**0.9670**	0.9371	0.9419	0.9598	0.9610	0.6066	15.3437
LOX08	0.9240	0.8664	0.8911	**0.9500**	0.8932	0.6109	9.9547
LOX09	0.8963	0.8215	0.8741	**0.9204**	0.8565	0.5127	12.2218
LOX10	**0.9340**	0.8838	0.8981	0.9285	0.9096	0.5644	13.0470
LOX11	0.9746	0.9503	0.9582	**0.9844**	0.9768	0.7490	13.7751
LOX12	0.9692	0.9361	0.9641	**0.9860**	0.9727	0.6634	17.1903
LOX13	**0.9797**	0.9222	0.9480	0.9604	0.9616	0.6613	17.6939
LOX14	**0.9672**	0.9416	0.9179	0.9401	0.9587	0.6785	13.9232
LOX15	**0.9582**	0.9291	0.9169	0.9276	0.9506	0.6453	15.2203
LOX16	0.9555	0.8923	0.9575	**0.9761**	0.9412	0.5809	18.5326
LOX17	0.9353	0.8527	0.8852	**0.9417**	0.8901	0.6358	10.6651
LOX18	0.9513	0.8928	0.9208	**0.9597**	0.9249	0.6197	12.5847
LOX19	**0.9425**	0.8612	0.9059	0.9374	0.9016	0.5895	14.7264
LOX20	**0.9746**	0.8308	0.9138	0.9715	0.9033	0.7076	12.0618
LOX21	0.9586	0.8676	0.9340	**0.9811**	0.9180	0.6881	12.0935
LOX22	**0.9769**	0.8998	0.9233	0.9732	0.9394	0.6922	11.5430
LOX23	**0.9634**	0.8881	0.8932	0.9531	0.9221	0.6859	10.0106
LOX24	**0.9483**	0.8970	0.8694	0.9209	0.9186	0.7175	10.9664
LOX25	**0.9720**	0.9274	0.9170	0.9508	0.9516	0.6609	13.3600
LOX26	**0.9541**	0.8995	0.8697	0.9438	0.9218	0.7179	8.2398
LOX27	**0.9501**	0.9010	0.8658	0.9452	0.9209	0.7283	7.5699
LOX28	**0.9431**	0.9089	0.8792	0.9150	0.9242	0.6151	9.7895

EOP04 showed the highest release rate, i.e., 84.5% of the drug in 12 hr as compared to other formulations (Figure 9).

An elementary osmotic pump Lornoxicam tablet (EOP) was taken for comparison with the controlled porosity osmotic pump Lornoxicam tablet (CPOP) and dissolution studies were observed. In the comparative study of the CPOP tablet and EOP tablet containing the same proportion of osmogent (sodium chloride) and when the release profile of CPOP formulation was compared with EOP formulation it was found that the CPOP formulation showed significantly higher release as compare to the EOP formulation. The comparison of CPOP and EOP tablets is shown in Figure 10.

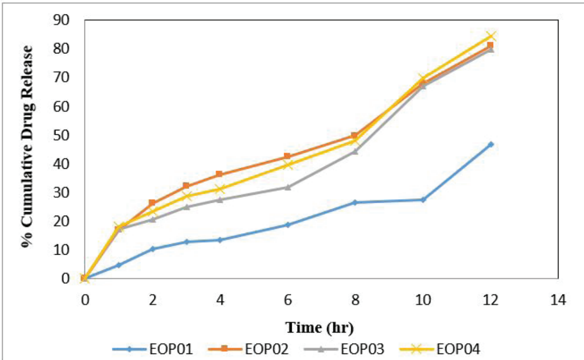

Figure 9. Comparison of *in vitro* release of batches EOP 01 to 04.

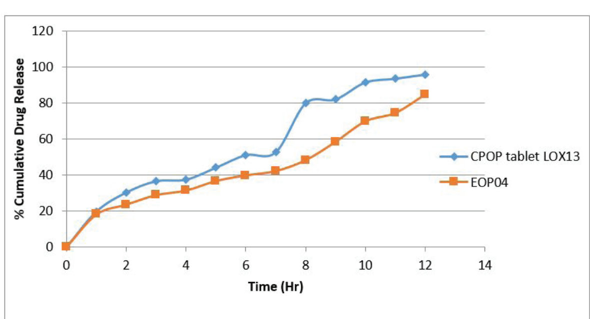

Figure 10. Comparison of *in vitro* release of batches EOP04 to CPOPLOX13.

In the EOP tablet, there is a chance of orifice blockage, which requires an extra formulation stage to increase the amount of drilling (for creating the orifice), which is time-consuming. Hence, CPOP is easier and more cost-effective to formulate. Based on the above, it is concluded that CPOP is superior to conventional EOP.

Statistical analysis

The release rate of different formulations was compared using one-way ANOVA. The statistical analysis was performed using Statistical Package for Social Science (SPSS) version 11. It was found that all the formulations were statistically significant $P<0.01$.

CONCLUSION

An osmotically regulated oral drug system (OODS) is suitable for the controlled release of drugs throughout the GI tract. A controlled porosity osmotic tablet is a novel concept in OODS, which is cost-effective and easy to formulate. In the present study, a controlled porosity osmotic (CPOP) tablet and an Elementary osmotic pump (EOP) tablet of Lornoxicam were formulated. The study involved the formulation and development of the CPOP and EOP and evaluation for various parameters like concentration of osmogent (NaCl, Mannitol), weight gain (percentage coating), and concentration of pore former (sorbitol). Drug release was directly proportional to the initial level of pore former (sorbitol), i.e., pore former increases with an increase in the release of the drug. The increase in lornoxicam release is due to the formation of more pores after coming in contact with an aqueous environment. The conclusions arrived at in this study indicated that the controlled porosity osmotic pump and Elementary osmotic pump tablet of Lornoxicam developed in this investigation were found to be a better-controlled.

Finally, it can be concluded that an osmotically controlled drug delivery system can control the release of Lornoxicam for 12 hours with a zero-order release kinetics, which can reduce dosing frequency and increase patient compliance and it will be a promising tool for better oral administration.

Abbreviations

OODS: Osmotically regulated oral drug system;

SPSS: Statistical Package for Social Science;

OPT: Osmotic pump tablet;

CDDS: controlled drug delivery systems

DOE: Design of experiment;

ANOVA: Analysis of variance;

DSC: Differential scanning colorimetry;

FTIR: Fourier transform infrared;

EOP: Elementary osmotic pump lornoxicam tablet;

CPOP: Controlled porosity osmotic pump lornoxicam tablet;

SEM: Scanning Electron Microscopy

CA: Cellulose acetate

Peer-review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- S.M.S., I.A.S., R.P.M.; Data Acquisition- S.M.S., I.A.S., R.P.M.; Data Analysis/Interpretation- S.M.S., I.A.S., R.P.M.; Drafting Manuscript- S.M.S., I.A.S.; Critical Revision of Manuscript- R.P.M.; Final Approval and Accountability- S.M.S., I.A.S., R.P.M.

Conflict of Interest: The authors have no conflict of interest to declare

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Original Article

Development of quantification technique for multiconstituent phytoformulation with recap of effects of combination therapy

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ABSTRACT

Background and Aims: The objective of this study was to extensively review therapeutic effects of co-administration of flavonoid curcuminoids and alkaloid piperine. Furthermore, the aim of this study was to develop a simple isocratic reversed-phase HPLC (RP-HPLC) method to quantify curcuminoids and piperine in the combined dosage form developed in-house.
Methods: The RP-HPLC quantification was performed on Inersil ODS-3V, 150 mm × 4.6 mm, 5 μm column using Acetonitrile: Buffer (35: 65 % v/v) as a mobile phase, at a flow rate of 1.5 mL/min. The curcuminoids and piperine were detected at wavelengths of 420 nm and 342nm, respectively. The method was validated according to the International Council for Harmonization (ICH) guideline Q2(R1).
Results: The individual curcuminoids and piperine peaks had theoretical plate number (N) > 4000 and a tailing factor (T) < 1.5 confirming well separation of the compounds. The calibration curve was linear from 0.6-18 μg/mL and 0.2-6 μg/mL for curcuminoids and piperine, respectively, with the correlation coefficient of >0.9990. The recovery and precision study values were in close agreement. The method was robust with relative standard deviation (RSD) less than 2%.
Conclusion: The literature survey indicated that the co-administration of piperine had influenced pharmacodynamic and pharmacokinetic activities of curcuminoids. The analytical method developed was found to be specific, sensitive, precise, and accurate for the estimation of curcuminoids and piperine in a single run.

Keywords: Co-administration, curcuminoids, piperine, RP-HPLC, validation

INTRODUCTION

For thousands of years, traditional medicines have been used in all parts of the world. Even today, they are used as a sole, complementary or alternative medicine. Most of them are polyherbal formulations containing number of herbs where herbs act as therapeutic agents and /or excipients. Variation in phytochemical composition of herbs and medicines is observed due to variation in genetic, seasonal, and geographical as well as herb processing methods. This sometimes results in poor quality of medicines with inferior or no therapeutic effect. Due to the complexity of chemical constituents, standardisation of herbal medicine is difficult. Thus, modern tendency is to isolate the therapeutic constituents and incorporate them in a dosage form. Isolation of morphine from opium is considered as the beginning of the new era. The number of isolated phytoconstituents is increasing day by day. (Krishnamurti, & Rao, 2016) Here, the preference is for the herbs which are used as a food which gives indication of safety. Also, these herbs are commercially cultivated, which ensures continuous availability of them. Turmeric is one such herb that is used as a spice in food and grown in many parts of the world with established medicinal values. It is often combined with black pepper in traditional medicines.

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Curcuma longa L. (turmeric) is a perennial plant of Zingiberaceae family. It grows around 1 meter. The rhizomes are cylindrical, yellow to orange, and highly branched. Rhizomes are washed, boiled, dried, and stored. As a spice, it is frequently ground to fine powder, and then used. As a home remedy, it is used for cuts, bruises, burns, swollen joints, skin diseases etc. It is taken internally as well as applied externally. The medicinal properties of the turmeric are attributed to the group of three yellow compounds (Figure 1) viz. curcumin, demethoxycurcumin and bisdemethoxycurcumin, collectively termed curcuminoids (Li et al.,2011). As most of the researchers find it difficult to isolate curcumin from the rest, three compounds are used as a mixture. Among curcuminoids, curcumin is in the highest quantity in the mixture, and in general, the mixture is simply referred as curcumin. Black pepper is the berry of trailing vine *Piper nigrum* L., family Piperaceae. Almost- ripened peppercorns are left to dry until they turn black. These are then ready to be consumed. Pungency and heat of the pepper is attributed to the piperine (Figure 1) found in it. People take black pepper orally for upset stomach, bronchitis, colic etc., and apply it on skin for discoloured skin (vitiligo) and nerve pain (Tasleem, Azhar, Ali, Perveen, & Mahmood, 2014).

Figure 1. Chemical structures of curcuminoids and piperine.

Effects of co-administration of curcuminoids and piperine

Solubility and permeability of curcuminoids

Curcumin is a symmetric molecule. It has an o-methoxy phenolic group attached to an aromatic ring. Two such aromatic rings are joined by a chain of seven carbon atoms carrying α, β-unsaturated β-diketone moiety. With such structure, hydrogen bonding happens between the two curcumin molecules. The process repeats, and curcumin molecules get stacked. Probability of hydrogen bonding between curcumin and water reduces significantly. Hence, curcumin is a highly hydrophobic and poorly soluble compound. Piperine gets incorporated in stacked layers of curcumin as it has competing hydrogen bonding forces. This reduces hydrophobicity of curcumin and increases quantity of curcumin in solution. This solubilized curcumin become available for absorption (Patil, Das, & Balasubramanian, 2016).

Piperine was found to increase skin permeability to curcumin in proportion to its concentration. A transdermal double layered patch of curcumin and piperine was developed. Piperine containing layer was kept in contact with skin. Thus, skin received pretreatment with piperine before receiving curcumin. An increase in permeation of 1.89 times was observed with piperine at a concentration of 7.41% of composite membrane (Jantarat, Sirathanarun, Boonmee, Meechoosin & Wangpittaya, 2018).

Pharmacokinetics of curcuminoids

Following its oral administration, curcumin is poorly absorbed through GI tract. The oral bioavailability in rats was about 1%. A fiftyfold higher oral dose of curcumin failed to simulate serum concentrations as that of intravenous administration (Yang, Lin, Tseng, Wang, & Tsai, 2007). Curcumin metabolism was studied by using isolated hepatocytes. Curcumin, after metabolism in the liver, is mainly excreted through bile. Curcumin shows two phases in metabolism. Curcuminoids undergoes several reactions of reduction by a reductase in phase I of metabolism. In phase II of metabolism, both curcuminoids and its phase I metabolites become water soluble by conjugation with glucuronic acid and sulphate (Pan , Huang, & Lin ,1999; Holder, Plummer, & Ryan 1978) These conjugates are excreted through urine. Thus, only a small fraction of oral curcuminoids become available for pharmacological actions, which making curcuminoids poor therapeutic agents.

Piperine acts as a bioenhancer *via* multiple pathways. It enhances bile acid secretion. Bile acid forms micelles of lipid and lipid soluble compounds that promote its absorption. Upon oral administration, it increases intestinal brush border membrane fluidity and microvilli length, which results in increased intestinal permeability of compounds (Khajuria, Thusu, & Zutshi, 2002). It influences P-glycoprotein (p-gp) efflux pump (Han, Tan, & Lim, 2008). In most of the situations, P-gp efflux pump of intestinal epithelium decreases intracellular concentration of compounds by transporting it back to the lumen of the intestine. Inhibition of P-gp efflux pump by piperine helps to retain absorbed curcumin. Oral administration of piperine in rats strongly inhibits the metabolic enzymes such as hepatic arylhydrocarbon hydroxylase and UDP-glucuronyltransferase. This in turn maintains concentration of original unmetabolised drugs in the body (Atal, Dubey, & Singh J, 1985). In rats, oral dose of curcumin and piperine at 2 g/kg and 20 mg/kg, respectively increased bioavailability of curcumin by 154%-fold compared to curcumin alone. In humans, co-administration of 2g of curcumin and 20 mg of piperine enhanced curcumin bioavailability by 2000% compared to curcumin alone (Shoba et al., 1998).

Pharmacodynamics of curcuminoids

Curcumin is cardioprotective in nature. In an experimental set up, cardiotoxicity was induced in rats by administration of cyclophosphamide. Curcumin and piperine at 50 mg/kg and 20 mg/kg respectively, showed extremely significant ($P < 0.001$) to moderately significant ($P < 0.01$) influence on parameters of cardiac health compared to curcumin (200 mg/kg) alone-treated group. The studied parameters included lipid profile, antioxidant levels, electrocardiogram, and histopathological score (Chakraborty, Bhattacharjee & Kamath, 2017). The hypocholesterolemic effects of curcumin were potentiated by co- administration of piperine. With the help of a high-fat diet, cholesterol levels were increased in Sprague-Dawley rats. The study indicated the enhanced activity and gene expression of apolipoprotein AI, lecithin cholesterol acyltransferase, cholesterol 7 alpha-hydroxylase, and low-density lipoprotein receptor (Tu et al., 2014). The radioprotective effects of curcumin and piperine alone and in combination were assessed on ionizing radiation exposed human normal lymphocytes. An additive radioprotective effect was observed in combination treatment at low concentration, though at higher concentration observations were not the same (Ghelishli, Ghasemi, & Hosseinimehr, 2019). Pretreatment with curcumin (100 mg/kg) and piperine (20 mg/kg) separately as well as in combination were given to male Swiss albino mice, which received benzo(a) pyrene, a known genotoxic. Combination treatment exerted potent antigenotoxic effect compared to curcumin alone (Sehgal, Kumar, Jain, & Dhawan, 2011). The combined treatment of curcumin (100 mg/kg; p.o.) with piperine (20 mg/kg; p.o.) showed better suppression of diethylnitrosamine induced hepatocellular carcinoma in comparison to curcumin alone. The findings were based on the morphological, histopathological, biochemical observations of the liver, and serum of rats (Patial et al.,2015). The combination chemotherapy of curcumin and piperine for colorectal carcinoma in HCT116 cells was studied. 1.5 times enhancement in apoptosis was observed when HCT116 cells were treated with a combination of CurcuEmulsome and PiperineEulsomes compared to CurcuEmulsome alone (Bolat et al.,2020). Glioblastoma multiforme is aggressive brain cancer with low life expectancy in patients. In cellular studies, IC_{50} of only curcumin and only piperine loaded gold nanogel were recorded at 30 µM and 35 µM, whereas gold nanogel containing the combination of both agents showed IC_{50} at 21 µM (Javed, Zhao, Cui, Curtin, & Tian, 2021).). Prophylactic antimalarial study was carried out in *Plasmodium berghei* ANKA-infected mice, and curcumin at dose of 300 mg/kg in combination with piperine 20 mg/kg showed delayed onset of clinical symptoms, and survival rate was prolonged compared to the treatment of individual drugs (Khairani et al., 2022).

In a clinical trial carried out on 44 osteoarthritic patients in Mumbai, a combination of curcumin 500 mg and piperine 5 mg was administered to the patients orally twice daily for 12 weeks. At the end of the study, statistically significant reduction in pain and stiffness in patients who received a combination of curcumin and piperine was observed. The need for additional NSAIDs for pain management also reduced (Reddy, & Faruqui 2016).

Based on these literature inputs, we aimed for the development of a gel formulation containing curcuminoids and piperine in the laboratory. The gel formulation contained excipients like polysorbate 80, Kolliphor RH 40, Kollisolv PEG 400, isopropyl alcohol, Carbopol 974P, sesamol, eucalyptus oil, triethanolamine, and purified water. For any pharmaceutical product development, assays form the integral part of the process. An analytical method was developed to estimate concentration of curcuminoids and piperine by using HPLC. HPLC is the most popular technique in pharmaceutical industry as it offers great flexibility in the procedure. This technique needs to be validated before its routine use. The present article further discusses the validation of the HPLC method.

MATERIALS AND METHODS

The curcumin (total curcuminoids 97%) was purchased from Sigma-Aldrich Chemie GmbH. Further curcuminoids were extracted in– house and were found to contain total curcuminoids 98.30%. This was used in the formulation and analysis. The standard piperine (97.1%) was purchased from Sigma-Aldrich Chemie GmbH. HPLC grade water was purchased from J.K. Labs, Thane, Maharashtra. Analytical grade orthophosphoric acid was obtained from Loba-Chemie, India. Methanol and Acetonitrile of HPLC grade were purchased from E. Merck, India.

Chromatographic system

The HPLC analysis was carried out on Agilent 1260 Infinity II (Agilent Technologies Deutschland GmbH, Wald Bronn Germany) equipped with UV/Vis Detector. The mobile phase was Acetonitrile: Buffer (35: 65 % v/v), where buffer was prepared by adding 0.1 % orthophosphoric acid (OPA) in water. The analysis was performed in isocratic mode with flow rate of 1.5 mL / min. 100 µL test solution was injected in the system. Separation was carried out on Inersil ODS-3V(150 mm X 4.6 mm, 5 µm) column by maintaining column oven temperature 40°C. The detection was carried out using UV-Vis detector at wavelength of 420 nm for curcuminoids and 342 nm for piperine. Total run time was 50 mins. Schematic diagram of the whole procedures is depicted in Figure 2.

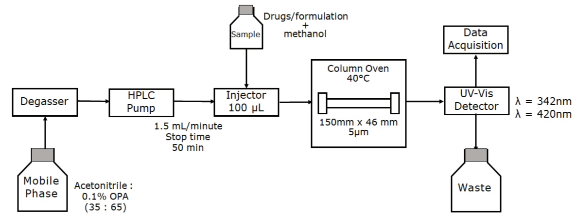

Figure 2. Schematic diagram of the HPLC system with parameters.

Stock and working solutions

Curcuminoids (5 mg) were weighed and transferred to volumetric flask. With sonication, it was completely dissolved in methanol. The volume was made up to get to the stock solution of 500 µg/mL curcuminoids . Similar procedure was followed to get the stock solution of 250 µg/mL piperine . 0.6 mL of curcuminoids stock solution and 0.4 mL of piperine stock solution were

transferred in 25 mL volumetric flask. The volume was made up to the mark with mobile phase to get the solution containing 12 μg/mL of curcuminoids and 4 μg/mL of piperine.

The required quantity of the test sample was weighed in a 50 mL beaker, and about 30-35 mL methanol was added. It was sonicated for 15 minutes with intermittent stirring every 5 minutes. The solution was transferred to a volumetric flask, and the volume was made up with methanol. The solution was filtered through 0.45μ Nylon syringe filter discarding 3-5 mL of sample. 1.6 mL of filtrate was diluted to 10 mL with mobile phase and mixed well to get the solution containing 12 μg/mL of curcuminoids and 4 μg/mL of piperine.

System suitability

Specificity of the analysis was established by injecting the mobile phase as well as the placebo solution that was obtained after giving the same treatment as the product into the HPLC system. Six replicate injections of the sample containing a mixture of curcuminoids and piperine at a concentration of 12 μg/mL and 4 μg/mL were analysed. Retention time (RT), asymmetry, theoretical plates, and resolutions were determined.

Linearity

Solutions of 6 different concentrations were prepared by taking 5% to 150 % of working concentration. Each level injected in triplicate. Linearity graph was plotted as concentration against mean peak area. Intercept, slope, and regression coefficient were calculated. The limit of detection and limit of quantification were calculated using the formula LOD = 3.3 Q/S and LOQ = 10 Q/S where Q is the standard deviation of the intercepts, and S is the slope of the calibration curve.

Accuracy

Accuracy of the proposed method was confirmed by spiking the placebo at 50%, 100%, and 150% concentrations, and then analysing it to determine the amount of the drug recovered.

Precision

Precision study was carried out in terms of repeatability (intraday precision) and inter-day precision. The gel containing curcuminoids and piperine was repeatedly analysed. Intraday precision study was carried out by estimating corresponding responses three times on the same day. For inter-day precision study, analysis was carried out by another analyst on another day in the same laboratory. The precision of proposed method was obtained by calculating the relative standard deviation (RSD) values observed for intra-day and inter-day analysis with acceptance criteria of RSD less than 2%.

Robustness

Robustness indicates the capacity of an analytical method to remain unaffected by minor changes in the method parameters. Effects of variation in experimental conditions like column temperature (± 2°C) and flow rate (± 10%) were evaluated.

RESULTS AND DISCUSSION

In reverse phase HPLC analysis, stationary phase is hydrophobic in nature, and accordingly, it retains hydrophobic molecule for longer time. Ionised species are comparatively hydrophilic in nature and are eluted fast. Elution of compounds is also affected by polarity of mobile phase. An increase in the polarity of the mobile phase results in longer retention times. Thus, careful selection of mobile phase is essential for good separation of compounds. Curcumin, demethoxycurcumin, and bisdemethoxycurcumin differ in the number of methoxy groups. They have molecular weights 368.38, 338.35, and 308.33, respectively. They have 3 labile protons and correspondingly have 3 pKa values. The first pKa is reported to be in the pH range of 7.5 to 8.5 (Priyadarsini, 2014). Piperine has a molecular weight of 285.34, and pKa of 12.22 (Lide, 2007). At a given pH, these compounds ionise to different extents because of the inherent structural difference. We optimised composition of mobile phase so that 3 curcuminoids and piperine were retained on the stationary phase for different time and eluted at different time without overlapping. We fixed it to acetonitrile: 0.1 % OPA in water at the ratio of 35: 65 % v/v.

The developed method was specific with no interference with placebo or mobile phase. The analysis was carried out in isocratic mode at flow rate of 1.5 mL / min, and the method was found to be reproducible. The phytoconstituents were analysed at two different wavelengths 420 nm for curcuminoids and 342 nm for piperine, and the detection specificity enhanced (Figure 3). The peaks of piperine, bisdemethoxycurcumin, demethoxycurcumin, and curcumin had good symmetry and were well-separated (Table 1). The method was found to be linear for curcuminoids and piperine in the range of 0.6 to 18 μg/mL and 0.2 to 6 μg/mL, respectively (Figures 4 and Figure 5). The LOD and LOQ for curcuminoids were 0.03523 and 0.1067 μg/mL, and for piperine 0.01277 and 0.0387 μg/mL, respectively.

Accuracy was studied in terms of recovery by spiking placebo at levels of 50%, 100% and 150% of the phytoconstituents. For recovery studies, % RSD was less than 1 (Tables 2 and Table 3). Similarly, intraday and inter day variation was found to be optimum with % RSD less than 1 (Table 4). The change in flow

Table 1. System suitability.

Name	Retention time	Asymmetry	Theoretical Plates	Resolution
Piperine	23.78 ± 0.01	1.02± 0.006	5746± 16.82	0
Bisdemethoxycurcumin	27.25 ± 0.031	1.29 ± 0.012	5054.67 ± 83.63	2.47± 0.01
Desmethoxycurcumin	31.50 ± 0.006	1.06 ± 0.012	5037.67 ± 42.59	2.58 ± 0.01
Curcumin	36.34 ±0.01	1.04 ± 0.015	4989.33 ± 27.47	2.52 ± 0.006

(Data given in this table is presented as mean ± SD, n=3, SD = Standard Deviation)

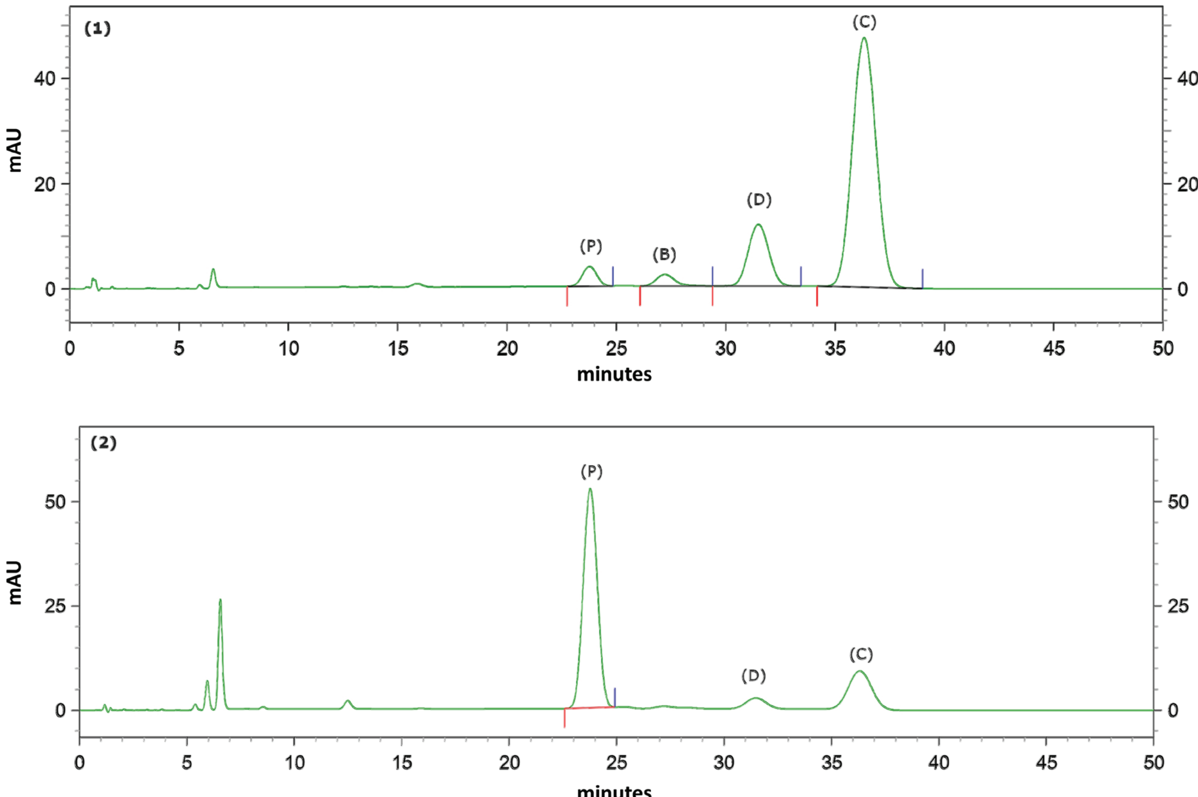

Figure 3. Chromatographic separation of curcuminoids and piperine with an isocratic mobile phase composed of an acetonitrile: buffer (35: 65 % v/v) at flow rate of 1.5 ml/min at (1) 420 nm and (2) 342 nm. Peaks representation (P)=piperine, (B)=bisdemethoxycurcumin, (D)=demethoxycurcumin, (C)= curcumin.

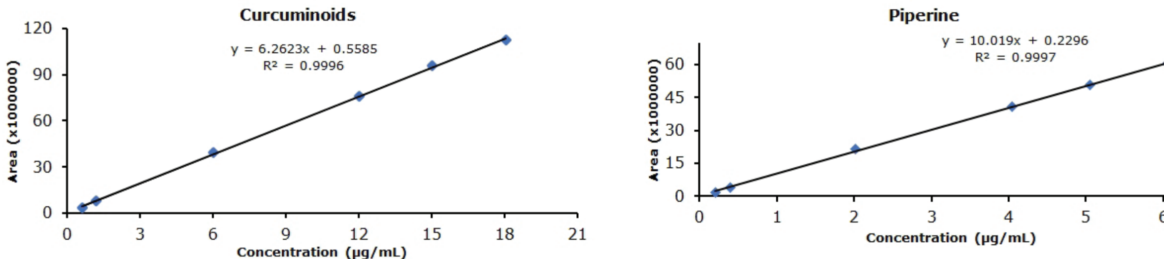

Figure 4. Linearity plot of curcuminoids. (Data given in mean ± SD) (n=3).

Figure 5. Linearity plot of piperine. (Data given is mean ± SD) (n=3).

Table 2. Recovery results for curcuminoids (acceptance limit recovery %= 98-102%).

Level in (%)	Amount spiked (μg/ml)	Amount recovered (μg/ml)	% Recovery	%RSD
50%	5.90	5.92	100.31	Mean= 100.37 % RSD=0.153
50%	5.90	5.91	100.25	
50%	5.90	5.93	100.54	
100%	11.80	11.81	100.12	Mean= 100.43 % RSD= 0.997
100%	11.80	11.75	99.62	
100%	11.80	11.98	101.55	
150%	17.69	17.70	100.02	Mean= 100.05 % RSD=0.211
150%	17.69	17.67	99.85	
150%	17.69	17.74	100.27	

rate by 10% and change in temperature by 2°C did not affect the analysis (Table 5). Good resolution, peak symmetry, and reproducibility outweighed its long run time.

As per USP 36-NF 31 curcuminoids are defined as "a partially purified natural complex of diarylheptanoid derivatives isolated from Turmeric, *Curcuma longa* L. It contains NLT 95.0% of curcuminoids, calculated on the dried basis, as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin." Also, as the dosage forms like capsule, USP directs that it should contain NLT 90.0% and NMT 110.0% of the labelled amount of curcuminoids, calculated as the sum of curcumin, desmethoxycurcumin, and bisdesmethoxycurcumin. Hence, it is important that an analytical method should detect and quantify individual three curcuminoids.

Scientists have used a mixture of curcuminoids for studies. Nowadays, they work on the effects of curcumin, demethoxycurcumin, and bisdemethoxycurcumin individually (Sato et al., 2014; Naksuriya, van Steenbergen, Torano, Okonogi, & Hennink, 2016; Liu et al 2021; Kumar, Lal, Nemaysh, & Luthra, 2018). There is a scope to use individual curcuminoids in combination with piperine. This underlines also the need to have an analytical method which can detect individual curcuminoids with piperine.

Analytical methods and validations are reported for quantification of curcumin and piperine in plasma. (Sethi et al., 2009; Rodriguez et al., 2021). Content of curcumin & piperine, and rutin, quercetin, curcumin, and piperine was estimated by RP-UFLC (Ramaswamy et al., 2014; Ramaswamy, Gowthamarajan, Dwarampudi, Bhaskaran, & Kadiyala, 2021) and RP- HPLC (Kuber,

Table 3. Recovery results for piperine (acceptance limit recovery%= 98-102%).

Level in (%)	Amount spiked (μg/ml)	Amount recovered (μg/ml)	% Recovery	%RSD
50%	2.01	2.00	99.22	Mean =99.75 %RSD= 0.526
50%	2.01	2.02	100.27	
50%	2.01	2.01	99.75	
100%	4.03	4.04	100.42	Mean =100.35 %RSD= 0.394
100%	4.03	4.02	99.92	
100%	4.03	4.06	100.70	
150%	6.04	6.03	99.83	Mean =99.60 %RSD= 0.296
150%	6.04	6.02	99.71	
150%	6.04	6.00	99.27	

Table 4. Precision studies for the developed method.

	Intra-day		Inter-day	
	% Curcuminoids	% Piperine	% Curcuminoids	% Piperine
Sample 1	99.29	98.23	99.56	100.19
Sample 2	98.87	98.33	99.73	99.92
Sample 3	99.00	99.43	101.04	98.79
Mean	99.06	98.66	100.11	99.64
SD	0.214885	0.668233	0.810486	0.743026
%RSD	0.217	0.677	0.810	0.746

Table 5. Robustness studies for the developed method.

Robustness Parameter	Level	Assay		% RSD	
		% Curcuminoids	% Piperine	Curcuminoids	Piperine
Flow rate	+10%	100.48	98.54	0.31	0.64
	-10%	100.30	99.81	0.30	0.79
Temperature	+2°C	100.07	100.14	0.78	0.70
	-2°C	100.24	99.95	0.04	0.44

(Data given in this table is presented as mean where n=3, RSD = Relative Standard Deviation)

2018). HPLC analytical method was developed for estimation of curcumin and piperine in nanoparticulate dosage form (Khismatrao, Bhairy, & Hirlekar, 2018). Curcumin, piperine, and camphor in an ayurvedic formulation were quantified (Shaikh and Jain 2018). All the analytical method development and validation studies mentioned above were not separated individual curcuminoids. An analytical method was developed for simultaneous quantification of curcumin and piperine in a microparticulate formulation with linearity in the range of 1.25 to 15 µg/mL for piperine and 2.50 to 30 µg /mL for curcumin. Though they could detect three separate peaks for individual curcuminoids, the resolution was less than 2, and only content of curcumin was quantified for validation (Setyaningsih et al., 2021). Reviewer guidance document for validation of chromatographic methods released by Center for Drug Evaluation and Research (CDER) recommends chromatographic resolution to be greater than 2. In this context, the analytical method developed by us has many advantages in terms of specificity and sensitivity.

CONCLUSION

From the literature, it can be concluded that curcuminoids- piperine combination therapy has many advantages. More studies need to be carried out with respect dose and dosage form for various ailments. A validated HPLC method for the simultaneous quantification of curcuminoids (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) and piperine has been established in this study. Accurate, sensitive, and reproducible quantification was possible with the developed method.

Peer-review: Externally peer-reviewed.

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Conflict of Interest: The authors have no conflict of interest to declare

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Original Article

Thermosensitive *in situ* gel formulation and characterization studies of *Sambucus ebulus* L. extract

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ABSTRACT

Background and Aims: *Sambucus ebulus* L. has been used to treat inflammation-related gastrointestinal disorders, influenza, kidney ailments, lung diseases, rheumatoid arthritis, and snake and insect bites. Our study provides important ethnobotanical information about *S. ebulus* with the aim of developing a formulation with increased extract bioavailability, diminished side effects, and easy drug loading and dose adjustment as an effective local therapy for dermatologic diseases.
Methods: Twelve *in-situ* gels with *S. ebulus* were prepared as antifungal treatments in accordance with the cold method and formulated using poloxamer and hydroxypropyl methylcellulose (HPMC). The formulations were characterized in terms of pH, gelling capacity, swelling degree, spreadability, and rheological properties.
Results: Among the prepared *in situ* gel formulations, the Poloxamer 407 and 407-HPMC mixtures of P14H1 and P15 demonstrated acceptable gelation temperatures for dermal use.
Conclusion: Thermosensitive *in-situ* gels containing *S. ebulus* may be a viable alternative for treating fungal diseases.

Keywords: *Sambucus ebulus* L., *in-situ* gel, gelling system, topical antifungal

INTRODUCTION

The genus *Sambucus* L. belongs to the *Adoxaceae* family, which is comprised of 30 species worldwide, including two (i.e., *Sambucus nigra* L. and *Sambucus ebulus* L.) that have been recorded in Turkey (Scopel et al., 2007; Senica, Stampar, & Mikulic-Petkovsek, 2019). *S. ebulus*, some of whose common names are elderberry, dwarf elder danewort, dane weed, danesblood, dwarf elder/European dwarf elder, walewort, dwarf elderberry, elderwort and blood hilder, is a type of shrub that is widely distributed in southern and central Europe and southwest Asia, especially in Iran and Turkey (Shokrzadeh & Saravi, 2010). The rootsstem barks, aerial parts, leaves, flowers, and fruits of *S. ebulus* have long been used to treat different ailments (Vallès, Bonet, & Agelet, 2004; Cvetanović, 2020; Charlebois, Byers, Finn, & Thomas, 2010). Due to its extensive usage by Anatolian people for medicinal purposes, they have given *S. ebulus* the name *hekimana* (mother of the physician) (Jabbari et al., 2017; Yeşilada, Gürbüz, & Toker, 2014). The traditional uses of the *S. ebulus* fruit for treating nail fungus infections were reported for the first time by Demirci & Özhatay in 2012. The design of the current study was inspired by this information after reviewing the remarkable knowledge obtained from the countryside in Kahramanmaraş, Turkey. Topical drug delivery is recognized as an effective local therapy for many dermatologic diseases. Active antifungal agents are commercially available in classic dosage forms such as creams, ointments, gels, and pastes (Hudson, Langer, Fink, & Kohane, 2010).

The roots of *S. ebulus* have been used for rheumatic diseases and arthritis, while its leaves are used in preparations for improving liver and kidney function and kidney functions. Its fruit is effective as a laxative and immune modulator. (Cvetanović, 2020). Re-

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ports exist of *S. ebulus* being used as a sedative, antispasmodic, diuretic, emetic, and laxative for treating asthma, bronchitis, cancer, edema, epilepsy, fever, gut issues, head pain, neuralgia, psoriasis, rheumatism, wounds, throat aches, and tooth pains (Kültür, 2007; Kaileh et al., 2007; Passalacqua, Guarrera, & De Fine, 2007; Charlebois et al., 2010; Melikoğlu, Kurtoğlu, & Kültür, 2015). In folk medicine, *S. ebulus L* is the most commonly used species for rheumatism, which is often observed in northern Anatolia due to the high humidity (Yeşilada et al., 1999). The traditional medicinal uses of *S. ebulus* L. in Anatolia are listed in Table 1.

S. ebulus berries are rich in several important secondary metabolites such as anthocyanins (cyanidin-3,5-diglucoside, cyanidin-3-sambubioside-5-glucoside, cyanidin-3-O-sambubioside, and cyanidin-3-O-glucoside), flavonoids (isorhamnetin-3-O-β-D-glucopyranoside, isorhamnetin-3-O-rutinoside, hyperoside, and isoquercitrin), iridoid glycosides (sambulin A, sambulin B), lectins (ebulin), phytosterols, phenols, triterpenes, tannins, cardiac glycosides, and phenolic acids (caffeic acid derivatives, chlorogenic acid, ursolic acid; (Atay, Kirmizibekmez, Gören, & Yeşilada, 2015; Cvetanović, 2020; Kaya et al., 2019; Shokrzadeh & Saravi, 2010).

Numerous studies have investigated the biological activities of these plants with the intention of discovering new pharmaceutical applications (Table 2). As such, anti-inflammatory (Ahmadiani et al., 1998; Ebrahimzadeh, Mahmoudi, & Salimi, 2006; Yeşilada, 1997), anti-nociceptive (Ahmadiani et al., 1998; Ebrahimzadeh et al., 2006), antimicrobial (Rodino et al., 2015; Salehzadeh, Asadpour, Naeemi, & Houshmand, 2014), anti-herpes simplex (Zahmanov et al., 2015), antiulcerogenic (Yeşilada et al., 2014), antioxidant (Cvetanović, 2020; Hashemi, Ebrahimzadeh, & Khalili, 2019), antihypoxic (Kaveh, Mohamadyan, & Ebrahimzadeh, 2019), hypolipidemic (Ivanova, Tasinov, & Kiselova-Kaneva, 2014), and wound healing (Süntar et al., 2010) activities have been demonstrated.

Table 1. Traditional medicinal uses of *Sambucus ebulus* L. in Turkiye.

Province	Local name	Parts used	Use	Literature
Giresun	Yabani mürver, Yer mürveri, Yivdim, Yivdin	flower, leaves radix	digestion analgesic, itching rheumatism, hemorrhoids, diuretic	Karaköse et al., 2017
Rize	İnçiyi, Levor	leaves, fruit	rheumatism, hemorrhoids	Saraç, 2013
Ordu	Yivdin, Yer mürveri, Yabani mürver	leaves, flower, fruit, aerial parts	diuretic, expectorant, flu treatment, antiparasitic, purgative, rheumatism, skin diseases	Gül et al., 2016 Aydın et al., 2018
Trabzon	Livor, Levor	leaves, flower	wounds, hemorrhoids, analgesic	Sağıroğlu et al., 2012 Bozkurt et al., 2017
Artvin	Anzili	leaves, fruit, radix	rheumatism	Eminağaoğlu et al., 2017
Samsun	Yivdin, Sultan otu	leaves, flower	burn treatment, eczema, antifungal, wounds, expectorant, antipyretic, analgesic, rheumatism, anti-inflammatory, hemorrhoids, animal bites	Karcı et al., 2017
Sakarya	Livor, Şahmelek, Şahmelik, Yiğdin, Yiğdün	leaves, flower aerial parts	diuretic, rheumatism, burn treatment	Koyuncu et al., 2009 Göç et al., 2019
Edirne	Sultan otu, Bizga, Mülver	leaves, fruit, herb	rheumatism, hemorrhoids	Güneş, 2018
Kırklareli	Sultanotu, Pıyran, Piran Haptoyina,	leaves, stem	rheumatism, hemorrhoids, wounds, snake bites	Kültür, 2007
İzmit	Lor, Lüver, Piran, Sultan, Şahmelek	leaves, stem	antiparasitic	Kızılarslan, 2012
Balıkesir	Bodur mürver	flower	antipyretic	Güner et al., 2016
Çanakkale	Sultan otu	leaves	rheumatism	Tuzlaci, 2015
Adana	Ayı otu	leaves, flower	hemorrhoids	Güneş et al., 2017
Kahramanmaraş	Ayıboğan, Yir otu	seed	rheumatism, hemorrhoids	Demirci et al., 2012

Table 2. Traditional medicinal uses of *Sambucus ebulus L.*

Country	Parts	Use	Literature
France	flower	Diuretic	Wichtl & Bisset, 1994
	leaves	Digestion	Shokrzadeh et al., 2010
	fruit	Purgative	Rigat et al., 2018
	seed	Analgesic	Shokrzadeh et al., 2010
Belgium	flower	Diuretic	Wichtl & Bisset, 1994
Spain	herb	veterinary medicine	Rigat et al., 2018
	leaves	Expectorant	
	radix	snake bites	
	leaves	anti-inflammatory, burn treatment	
Iran	herb	analgesic, uterus diseases, burn treatment, dental infections, diuretic, purgative, animal bites	Jabbari et al., 2017
Bulgaria	fruit	diuretic, purgative, antiseptic	Kaya et al., 2019
	radix	Digestion	
Romania		rheumatism, diuretic	Chirigiu et al., 2011
Croatia	fruit	rheumatism, antipyretic	Pieroni et al., 2003
	seed / radix	liver diseases	Charlebois et al., 2010
Serbia	herb	anti-inflammatory, antioxidant	Popović et al., 2020
Germany	flower fruit	digestion, neuropathic pain, dental diseases	Bradley et al., 1992 Mahboubi, 2020
USA	flower	flu treatment, analgesic	Mahboubi, 2020

Topical treatments are frequently used for preventing or treating diseases locally. In this regard, gel systems have advantages such as high patient compliance and ease of use and preparation. For these reasons, drugs that can be topically applied are often preferred (Nirmal, Bakliwal, & Pawar, 2010). *In-situ* gel systems provide various advantages such as prolonged skin contact and ease of drug loading and dose adjustment. Biocompatibility of polymeric delivery systems is also clinically important (Kang & Singh, 2005). Gel systems can control and sustain drug release, which increases bioavailability, decreases side effects, reduces systemic absorption, and improves patient resistance by consolidating dosing frequency (Agrawal, Das, & Jain, 2012; Bhattacharjee, Beck-Broichsitter, & Banga, 2020).

In-situ gel formation involves several mechanisms, including pH change, ionic cross-linkage, and temperature modulation. Innovative polymeric systems represent a promising method of applying drugs, as these polymers transform from a solution into a gel state once administered (Nirmal et al., 2010; Khode & Dongare, 2019; Khule & Vyavahare, 2021). Thermosensitive polymers are probably the most studied class of environment-sensitive polymer systems in drug delivery research. A polymer is a solution at room temperature that gels at body temperature. Poloxamers usually have an efficient thermo-reversible property with a characteristic sol-gel transition temperature that is used widely in *in-situ* gelling systems (Güven, Berkman, Şenel & Yazan, 2019; Xie et al., 2019; Niyompanich, Chuysinuan, Pavasant, & Supaphol, 2021).

Topical delivery of *S. ebulus* is very suitable for antifungal treatment because the first-pass effect is preventable, and a topical application can directly target the skin. As a main physiological skin barrier, the stratum corneum limits the absorption of foreign materials into the body. (Güngör, Erdal, & Aksu, 2013; Rezaei-Moshaei et al., 2021). Therefore, the efficiency of topical treatment depends on penetration capability of the active agent through the *stratum corneum*. The formulation may play a key role in drug penetration into the skin, *in-situ* gel systems for topical antifungal treatment may improve skin penetration of active compounds (Hudson et al., 2010; Güngör et al., 2013) and, directly target lesions for maximum local therapeutic effect. Applying drugs in this way is beneficial for skin disorders where the treatment should ideally accumulate on the skin surface and not pass deeper so as to avoid systemic side effects (Erol et al., 2020).

This study proposes a new approach for the topical delivery of *S. ebulus* by employing *in-situ* gel formulations. The main aim is to develop and characterize a in situ gel formulation of *S. ebulus* that may have a potential to increase its topical efficacy and, to diminish systemic side effects.

MATERIALS AND METHODS

Chemicals and plant material

Poloxamer 407® and hydroxypropyl methylcellulose (HPMC; Sigma-Aldrich, Germany) are used as the *in-situ* gel formulation polymers. Glycerin (Sigma-Aldrich, Germany) is the preferred plasticizer. Benzalkonium chloride (Fluka, Germany) is used as the preservative, and distilled water has been pre-

ferred as the solvent. Plant material and fruit from *S. ebulus* have been collected from Andırın in Kahramanmaraş Province in June 2018. The voucher specimen was deposited at the Faculty of Pharmacy of Cukurova University Herbarium (CUEF 1671).

Extraction

The fresh fruit were divided into two groups. The first group was dried at room temperature in the shade, and then the dried material was extracted with a methanol-water (50:50; v/v) mixture using a shaker at 25°C for 24 hours. The procedure was repeated four times until the samples were exhausted. After filtration, the solvent was removed by rotary, and the water was removed by lyophilization. The extract (DFM) was stored at -20°C until analysis. The second group was squeezed.

Preparing the *in-situ* gel formulation

The *in-situ* gel formulations were prepared using the cold method (Güven et al., 2019). First, Poloxamer 407® was dissolved in distilled water at 13–20% (w/w) concentrations. Then, 1% (w/w) of the HPMC was added drop by drop into the polymer solution under magnetic stirring (500 rpm) at 4°C. The gels were left at 4°C until the poloxamer completely dissolved. Lastly, 0.005% benzalkonium chloride and 5% glycerin were added to the solution. The gel formulation compositions are provided in Table 3. All final formulations have been evaluated for their gelation temperature.

Physicochemical characterization of *in situ* gels

Formulation appearance

The clarity, visual appearance, and particle content of the gels were determined using optical tests under a dark background and rated as follows: turbid = +; clear = ++; and very clear = +++ (Okur, Yoltaş, & Yozgatlı, 2016). Accelerated stability studies were performed by exposing ideal formulations at various temperatures between 8 and 40°C. After a week of storage for assessing stability, the *in-situ* gel was then evaluated for its and physicochemical characteristics.

Gelation temperature measurement (Tsol-Gel)

To determine the gelation temperature, a glass test tube containing the gel formulation was put in a water bath (25°C) and warmed in 2°C increments. The temperature at which the flow of the formulation stopped upon turning over the container was measured as the gelation temperature. The temperature at which the solution was converted to a gel was determined using a thermometer placed in the test tube. The formulations were evaluated using the test tube tilting method between the temperatures of 25-40°C. The results are an average of three determinations (Xie et al., 2019). The study proceeded with the gelling systems that had undergone gelation at the appropriate temperature.

Measuring pH

The pHs of the prepared formulations were checked using a calibrated pH meter (WTW ProfiLab pH 597 Meter, Germany) at room temperature. All analyses were conducted in triplicate.

Gelling capacity

The formulations were dropped into 2 ml of distilled water, and the gelling time was visually recorded to determine gelling capacities. The code system on the table was used to determine the gel formation capacity (Yara, 2019).

Swelling Studies

Distilled water was used at 37±1°C for the formulation swelling studies. 1 ml formulation sample was put on a dialysis membrane and fixed to prevent leakage, after which the gel's weight was measured, noted, and reweighed after being kept in distilled water for a certain period. The following formulation was used to calculate the swelling rates (Güven et al., 2019):

Swelling ratio (t) = {[gel weight (t) - gel weight (t0)] / gel weight (t0)} x 100 (1)

Determining Rheological Properties

The rheology of the developed formulations was performed using a digital cone-plate rheometer (Brookfield, USA). The rheological tests were done at two different temperatures) (25±1°C and 37±1°C. Shear stress values were recorded by determining the difference in viscosity change and shear rate between the two temperature measurements as 0 and 2000 1/s (second) (Güven et al., 2019).

Table 3. Composition of the tested topical *in-situ* gel formulations.

Code / ingredients (%)	*Sambucus ebulus* L. extract	Poloxamer 407	HPMC	Glycerin	Benzalkonium chloride
P13	5	13	-	5	0.005
P13H1	5	13	1	5	0.005
P14	5	14	-	5	0.005
P14H1	5	14	1	5	0.005
P15	5	15	-	5	0.005
P15H1	5	15	1	5	0.005
P16	5	16	-	5	0.005
P16H1	5	16	1	5	0.005
P17	5	17	-	5	0.005
P17H1	5	17	1	5	0.005

Spreadability

Spreadability is the area over which the gel formulation diffuses per unit time (cm^2/min). The spreadability of the prepared formulations was determined using filter paper. A 1-ml calibrated pipette was fixed to the stand so that the last point of the pipette was 2 cm above the filter paper. The liquid formulation (0.1 ml) was dripped onto the middle of the filter paper. After a fixed period of time, the diameter of the surface area covered by the spreading of the formulation was determined and measured in triplicate (Chaudhary & Verma, 2014).

Accelerated stability

The optimized formulations were maintained at 40±2°C and 8±2°C for one month. Samples were withdrawn weekly and tested for pH, visual appearance, color, and gelling capacity (Mandal et al., 2012).

Statistical analysis

Data were shown as the mean ± *SD* (n = 3). Statistical data were analyzed using the Student's t-test with a significance level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Preparing the *in situ* gel formulation

The thermosensitive *in-situ* gel formulations that were prepared using the cold method included Poloxamer 407 and HPMC. The cold method is a formulation technique favorable for use because it provides transparent formulations for gel systems and does not form the polymer globs that occur when the hot method is applied (Choi et al., 1998).

Poloxamer 407 has been demonstrated to have low side effects, perfect solubility, good drug release characteristics, and conformity with other formulation ingredients. This study uses Poloxamer 407 for its temperature-dependent gelation properties as a base for the semisolid drug delivery system with suitable sol-gel temperatures (36–38°C; Liu et al., 2019).

Poloxamer 407 and HPMC were selected as the polymers, as these polymers provide prolonged residence time at the application site (Nirmal et al., 2010). The neutral polymer, HPMC, was selected for its mucoadhesive properties, good swelling characteristic, low toxicity, and low irritancy (Liu et al., 2019).

Physicochemical characterization of *in situ* gels

Formulation appearance

The thermosensitive *in-situ* gel systems were visually evaluated in terms of color, transparency, and dissolution state in both solution and gel form. All *in-situ* gels showed good homogeneity without globs, and all formulations had sufficient clarity (Table 4).

Determining solution gelation temperature (Tsol-gel)

The transformation from solution to gel after applying the formulation is important for thermosensitive *in-situ* gel formulations. The impact of polymers on gelation temperature is based on chemical characterizations and the concentration in the formulations. The formulations were therefore examined to determine whether they had reached a sol-gel temperature suitable for topical application (Yuan et al., 2012).

Solutions containing less than 13% Poloxamer 407 did not form a gel over the evaluated temperature ranges. The gelation temperatures of poloxamer solutions containing 13-17% Poloxamer 407 and P407-HPMC mixtures ranged from 25-40°C, with several formulations gelling at body temperature (Table 4).

Gelation temperatures above 38°C saw the formulation remain a liquid at body temperature. Gelation temperatures of the *in-situ* gel lower than 25°C saw gelation occur at room temperature, leading to difficulty administering to the diseased area. Therefore, *in-situ* gel formulations with an average gelation temperature between 32°C-35°C are preferred, as they are likely to spread easily at room temperature but gel rapidly when in contact with skin (Liu et al., 2019).

Measuring pH

A pH meter was used to measure pH values to investigate the compatibility of the developed *in-situ* gel formulations with dermal surfaces. The pH of these formulations ranged between 6.6-6.8 (Table 5), which is close to the natural pH of the skin surface. The results of the pH analysis showed that the tested *in-situ* gel formulations to be compatible with the skin and to be unlikely

Table 4. Evaluation of the *in-situ* gel formulation data.

Code	Clarity	T (sol-gel,°C)
P13	+++	sol state
P13H1	++	38.4 ± 0.2
P14	+++	35.4 ± 0.1
P14H1	++	34.2 ± 0.1
P15	+++	32.8 ± 0.2
P15H1	++	30.8 ± 0.1
P16	+++	28.2 ± 0.2
P16H1	++	26.5 ± 0.2
P17	+++	25.1 ± 0.2
P17H1	++	gel state
P18	+++	gel state
P18H1	++	gel state

Table 5. Physicochemical properties of the selected formulations.

Code	pH	Gelling capacity	Spreadability (cm^2/min)
P14H1	6.6 ± 0.2	+++	6.76 ± 0.60
P15	6.8 ± 0.2	+++	4.84 ± 0.80

to exert any local irritation or inflammation on pH-sensitive skin (Yara, 2019; Salatin, Lotfipour, & Jelvehgari, 2020).

Gelling capacity

Gelling capacities of the formulations have been visually scored according to the following grades: (-) no gelation, (+) gelation after a few minutes followed by fast dissolution, (++) immediate gelation remaining for a few minutes, and (+++) immediate gelation remaining for nearly an hour. A high gelling capacity was obtained with the evaluated formulations. The gelling capacity data of the prepared formulations reveal the formulations to have immediate gelation that could be maintained for nearly an hour (Table 5). The inclusion of HPMC improved the gelling capacity of the formulations compared to Poloxamer 407 alone.

The gelling capacity is based on gelation time and dissolution time of the formed gel due to environmental properties. Thus, by increasing the concentration of the polymer, the transition time was decreased, and the dissolution time of the formed gel was extended (Gugleva et al., 2020).

Swelling studies

The *in-situ* gels were observed to be stable throughout the period of swelling (6 hours). The rate of swelling from P14H1 was slower than from P15 (Fig. 1). The results show the *in-situ* gels to be less swollen than the poloxamer gel alone due to the formation of very hard gels when HPMC is combined with the poloxamer (Güven et al., 2019).

Determining the rheological properties

The rheological behaviors of semisolid formulations are important for dosing, flowability, drug release, and patient compliance with topical applications. The selected formulations have been subjected to rheological studies. The rheograms of the formulations are shown in Figure 2. The *in-situ* gels have been demonstrated to display pseudo-plastic flow and shear-thinning rheological behaviors. The *in-situ* gel systems should not have a high viscosity when administered to the skin. Once administered, the *in-situ* gel formulation is desired to have a high viscosity in order to maintain contact with the area for a more extended period of time so as to provide a therapeutic effect (Öz et al., 2020).

Developed formulations were Newtonian below the gelation temperature (25°C), but achieved pseudo-plastic flow upon reaching body temperature, as was expected due to their thermosensitive properties (Gugleva et al., 2020). The formulations showed characteristic rheological responses of hard gels at physiological temperatures. This thermosensitive formulation showed Newtonian flow at 25°C and a rapid rise in viscosity at 37°C.

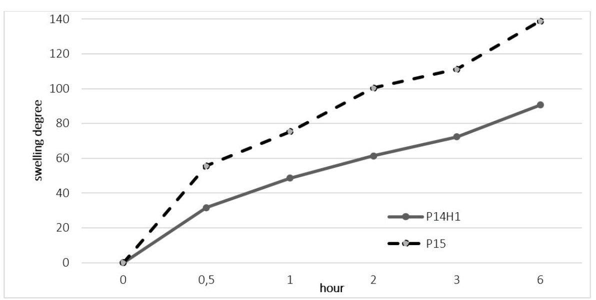

Figure 1. Swelling profiles of the selected formulations.

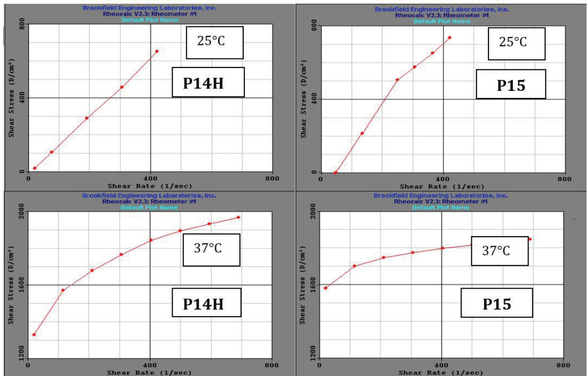

Figure 2. Rheograms of selected formulations (25°C and 37°C).

Spreadability

Spreadability is an important parameter for patient conformity and helps provide uniform gel application to the skin. High gel spreadability limits the time it can be spread on dermal surfaces. Spreadability refers to the distance traveled by the formulations before they transition to a gel. An increase in the poloxamer concentration reduces the spreadability of the formulations due to the reduced gelation temperature (Rençber et al., 2017). Table 5 shows the spreadability of formulations P14H1 and P15. Formulation P14H1 shows good spreadability compared to P15. Lastly, the two formulations both show good spreadability for topical application.

Accelerated Stability

Accelerated stability studies were performed by exposing the ideal formulations to various temperatures between 8 and 40ºC. After a week of storagef, the *in-situ* gel was evaluated for its appearance and physicochemical characteristics. No major changes were observed regarding its physicochemical characteristics or appearance.

CONCLUSION

The topical treatment of cutaneous infections is preferred compared to oral treatments. The benefits of topical drugs include avoiding systemic adverse effects, directly targeting the site of infection, and high patient compliance. *In-situ* gel systems have superiorities such as providing easy topical application, increased residence time and prolonging drug release. In this study *in-situ* gels with *S. ebulus* was prepared for topical antifungal treatment and, focused on the characterization of thermosensitive *in-situ* gels containing the *Sambucus* extract as designed for topical administration. Among the 12 different *in-situ* gel formulations prepared using mixtures of Poloxamer 407 and 407-HPMC, the P14H1 and P15 formulations are seen to demonstrate an acceptable gelation temperature for dermal use.

The formulations have been characterized in terms of pH, gelling capacity, swelling degree, spreadability, and rheologi-

cal properties. Based on the conducted study, the following conclusions have been drawn. The gels that were prepared by using the cold method with the *Sambucu*s extract show good physicochemical proportions. As a result, the Poloxamer 407-HPMC-based *in-situ* gel formulation containing the *Sambucus* extract was determined to be an efficient alternative for treating dermal fungal diseases. The developed formulation can extend the antifungal activity of the *Sambucus* extract for a longer period of time. Future controlled *in-vivo* trials are needed to evaluate the efficacy of the plant extract in formulations.

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Original Article

Biological and computational evaluation of carbazole-based bis-thiosemicarbazones: A selective enzyme inhibition study between α-amylase and α-glucosidase

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ABSTRACT

Background and Aims: Carbazole heterocyclic systems are an important class of chemicals that have been reported as valuable antidiabetic agents in the literature. Uncoincidentally, the ayurvedic antidiabetic plant *Murraya koenigii* Spreng (Curry tree) was the source of the first carbazole alkaloids. Another important class of chemicals in terms of antidiabetic activity is thiosemicarbazones. The hybridization of these fragments can create new potential inhibitors for α-amylase and α-glucosidase enzyme inhibitions, which is one approach controlling post-prandial hyperglycemia in type 2 diabetes patients.
Methods: The four carbazole-based thiosemicarbazone compounds (**4a-d**) have been selected from the group library and α-amylase and α-glucosidase inhibition potencies have been evaluated. A molecular modelling study has also been carried out to provide a complementary study on how the molecules behave in terms of the enzymes' catalytic properties. .
Results: All compounds showed higher potencies than the standard acarbose in terms of α-glucosidase inhibition and very low inhibitions toward α-amylase compared to acarbose. Having the number of hydrophobic interactions determine the potency of the compounds was crucial with compound **4a** being shown to provide the highest number of conventional H bonds and the highest percentage of inhibition values for both enzymes.
Conclusion: Carbazole-based thiosemicarbazone compounds have been found to be promising candidates in terms of both their potency and relative selectivity for developing new inhibitors that lack the usual side effects of current drugs.

Keywords: α-amylase, α-glucosidase, carbazole, molecular docking, thiosemicarbazones

INTRODUCTION

Natural compounds have been used as models to identify new active molecules in organic chemistry. 9H-Carbazole was discovered as an anthracene derivative in 1872 (Graebe & Glaser, 1872) and has been illustrated as an important heterocyclic compound model. Afterward, the interest in carbazole derivatives increased significantly through the strong antimicrobial effects of the alkaloid named murrayanine that was isolated from *Murraya koenigii* Spreng (Schmidt, Reddy, & Knölker, 2012a). Numerous studies conducted along these lines have identified carbazole derivatives as valuable therapeutic targets due to their antidiabetic, anti-

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HIV, antitumor, antibacterial, and neuroprotective potentials. After 150 years, many natural and synthetic carbazole derivatives are now available for different diseases including cancer in the pharmaceutical market (Tan, Sharma, & An, 2022).

The natural compounds and traditional medicines have been used for the discovery of new naturally active synthetic compounds.. For example, *M. koenigii*, from which the first bioactive carbazole alkaloids were obtained, is used to treat diabetes in traditional Indian medicine (i.e., Ayurveda). Many pharmacognostic studies conducted in this context have attributed the hypoglycemic effect of the plant to its carbazole derivatives (Kesari, Kesari, Singh, Gupta, & Watal, 2007; Patel et al., 2016). Non-insulin dependent type 2 diabetes patients constitute the majority of *diabetes mellitus* patients with altered blood glucose levels, and α-amylase and α-glucosidase inhibitors are used on these patients to prevent postprandial hyperglycemia and its complications. In this way, these enzymes are prevented from hydrolyzing polysaccharides in foods into monosaccharides. Currently, acarbose, miglitol, and voglibose are the active pharmaceutical compounds prescribed for this purpose. The search for new inhibitors continues due to the side effects (e.g., abdominal pain, nausea, vomiting and meteorism) from these non-selective inhibitors. Many studies are also found to have reported carbazole derivatives to be potent α-glucosidase inhibitors (Adib et al., 2019; Dhameja & Gupta, 2019; Iqbal et al., 2017).

Thiosemicarbazones are derived from the condensation reaction of aldehyde functionality and thiosemicarbazides and additionally have attracted great attention due to the presence of $R^1R^2C=N-NH-(C=S)-NR^3R^4$ moiety. The conjugated *N-N-S* system provides an important therapeutic potential to thiosemicarbazone-based systems, with interesting interactions occurring with the biomolecules (Antholine, Knight, Whelan, & Petering, 1977; Richardson, 2002; Shahabadi, Kashanian, & Darabi, 2010). Specifically, thiosemicarbazone fragments have been reported as important targets for antidiabetic studies. A new series of benzoxazinone-thiosemicarbazones have been reported as potent inhibitors of aldo-keto reductases, which are important enzymes for the polyol pathway (Shehzad et al., 2019). Tok et al. (Tok, Küçükal, Baltaş, Tatar Yılmaz, & Koçyiğit-Kaymakçıoğlu, 2022) reported on a range of thiosemicarbazones derived from substituted sulfonyl acetophenone and evaluated for α-amylase and glucosidase inhibition potency. They also carried out kinetic studies of the designated compounds and revealed their competitive mode of binding. A series of indole-based thiosemicarbazones have also been designed and tested for identifying aldose reductase (ALR2) and aldehyde reductase (ALR1) potency (Shehzad et al., 2021), which also concluded the reported compounds to display selective potential toward complications associated with *diabetes mellitus*.

As a result, this study aims to explore the antidiabetic potentials of hybrid molecules derived from carbazole and substituted thiosemicarbazides. The main consideration is to create multiple binding patterns between the designated compounds and different binding pockets on the α-amylase and α-glucosidase enzymes. The individual antidiabetic potentials of each fragment can help increase the inhibitory activity and provide better compounds as more promising inhibitors. Four compounds (**4a-d)** have been chosen from our groups` chemical library containing promising fragments (carbazole and thiosemicarbazones) and tested them against the responsible enzymes for an antidiabetic study. The computational study has also been carried out to understand the binding behaviors of the compounds in terms of the enzymes' catalytic sides. The practical results obtained from the bioassays have been compared with the theoretical data from the molecular docking study. This study is a complementary report that provides an important comparison of practical and theoretical data for antidiabetic study.

MATERIALS AND METHODS

Chemicals and physical measurements

The general synthetic procedure for the known compounds has been reported in the appropriate reference (Bingul et al., 2019). All commercially available reagents and standards, as well as the α-amylase type VI-B (E.C. 3.2.1.1 from porcine pancreas, lyophilized powder), 2-chloro-4-nitrophenyl-α-D-maltotrioside (CNP-G3), sodium chloride, sodium azide, acarbose, Dimethyl sulfoxide (DMSO), p-nitrophenol, α-D-glycopyranoside (PNPG), and α-glucosidase type I (E.C. 3.2.1.20, from *Saccharomyces cerevisiae*, lyophilized powder) as used for the biological assays were purchased from Sigma Aldrich (St. Louis, MO) and carried out without further purification. All other chemicals were of analytical grade.

The α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was performed using the method described by Schmidt et al. (Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012b). In brief, 90 µL of 0.1 M phosphate buffer (pH 7.5, 0.02% NaN_3), a 10 µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration of 0.05 U/mL) were added to each well. The mixture was incubated at 28 °C for 10 min before adding PNPG to a final volume of 200 µL (final well concentration of 1.0 mM). A blank was used consisting of the enzyme, substrate, and test solvent instead of the sample. Absorbance was measured at 405 nm every 40 s for 35 min. BioTek PowerWave XS microplate photometer with built-in incubator and controlled by the program GEN5 9ver. 2.05.20050 was used for the incubation and absorbance measurements. The α-glucosidase inhibitory activity was expressed as percentage inhibition and calculated using the following formula:

$$\text{Inhibition } \% = (\text{Slope}_{blank} - \text{Slope}_{sample}) / \text{Slope}_{blank} * 100 \quad (1)$$

Acarbose was used as the positive control, and all measurements were performed in triplicate (Student's t-test with $p < 0.05$ showing significance).

The α-Amylase inhibition assay

The α-amylase inhibitory activity was measured using the method described by Okutan et al. (Okutan, Kongstad, Jäger, & Staerk, 2014) with the minor changes. In brief, 80 µL of 0.1 M phosphate buffer (pH 6.0, 0.02% NaN_3), a 20 µL test sample dissolved in DMSO, and 80 µL of enzyme solution (well concentration of 0.05 U/mL) were added to each well. After incubation at 37°C for 10 min, the reaction was started by adding CNP-G3 to a final volume of 200 µL (final well concentration of 1.0 mM). A blank was used consisting of the enzyme, sub-

strate, and test solvent instead of the sample. Absorbance was measured at 405 nm at for 30m. A background sample consisting of enzyme, sample, and buffer in place of the substrate for each compound was employed to eliminate the effect of the absorbance for analytes that absorbs at 405 nm. The BioTek Power Wave XS microplate photometer with built-in incubator controlled by the program GEN5 (ver. 2.05.2005) was used for the incubation and absorbance measurements. The α-amylase inhibitory activity was expressed as percentage inhibition ± SD and calculated using the following formula:

$$\text{inhibition } \% = (A_{blank} - A_{sample}) / A_{blank} * 100 \qquad (2)$$

Acarbose was used as the positive control, and all measurements were performed in triplicate (Student's t-test with $p < 0.05$ showing significance).

Molecular modeling

Receptor preparation

The crystallographic structures of α-amylase and α-glucosidase were respectively obtained from the Protein Data Bank (Berman, et al., 2000) as 1B2Y (crystallographic structures of α-amylase, 3.20 Å) (Nahoum et al., 2000) and 3W37 (crystallographic structures of α-glucosidase1.70 Å) (Tagami et al., 2013). The crystal structures were cleaned from all ingredients contained in the PDB file apart from the amino acid residues using BIOVA Ds Visualizer. The program MGLTools was used to add missing residues, hydrogen atoms, and charges and to remove non-polar hydrogen atoms.

Ligand preparation

Three-dimensional structures of the synthesized ligands **4a-d** were drawn using the Biovia DS Visualizer (Biovia, 2019) and displayed using the Chimera (UCSF) software package (Pettersen et al., 2004). All molecules were firstly optimized using the semi-empirical AM1 method, with the optimization taking place using Gaussview 5.0.9 (Frisch et al., 2009). AM1-Bcc (the Austin model with bond and charge correction) atomic partial charges for the ligands were determined using the antechamber module of the package program AMBER v.11. (Jakalian, Jack, & Bayly, 2002).

Docking study

This research performed the docking studies using Dock 6.5. Grid generations were computed by centering a box to the binding sites of the receptors. Grid boxes were centered to the binding sites of the enzymes with the x, y, and z coordinates of 66.521, 51.261, and 12.259 for the α-amylase and 39.434, 33.431, 6.003 for the α-glucosidase, with the dimensions being defined as 40 40 40 Å for the enzymes. The Lamarckian genetic algorithm was used with a population of 300 individuals and docking settings of 2,500,000 maximum energy evaluations, and 54,000 maximum generations to give 250 runs.

RESULTS AND DISCUSSION

Chemistry

Synthesis of the Carbazole-Based Bis-Thiosemicarbazones 4a-d

Syntheses of the targeted carbazole-based bis-thiosemicarbazones **4a-d** were achieved using two reactions, starting with the Vilsmeier Hack formylation of *N*-ethly carbazole **1** in the presence of $POCl_3$ and DMF followed by the Schiff base condensation reaction of the corresponding carbazole 3,6-dicarbaldehyde **2** with the thiosemicarbazides **3a-d** under acidic conditions for good yields (Figure 1). The syntheses and characterization analyses (Fourier Transform Infrared) FT-IR, (Nuclear Magnetic Resonance) NMR, and (High Resolution Mass Spectroscopy) HRMS) of the final compounds **4a-d** have already been discussed in a previous study (Bingul et al., 2019).

Thiosemicarbazides	R_1	R_2	Carbazole-based bis-Thiosemicarbazones
3a	H	H	4a
3b	Me	H	4b
3c	Me	Me	4c
3d	Et	H	4d

Figure 1. Reagents, conditions and compounds: i) $POCl_3$, DMF, reflux, 24 h, ii) Thiosemicarbazides (3a-d), EtOH, CH_3COOH, r.t.

Biological studies

α-Amylase and α-Glucosidase inhibitory activity

Table 1 shows the α-glucosidase and α-amylase inhibition potencies of the four compounds **4a-d** at the 800 µM concentration, with acarbose being used as the standard. The inhibition concentrations revealed the compounds to be more potent than the standard acarbose in the case of α-glucosidase. The % inhibition value of acarbose against α-glucosidase was determined as 70.95% at 1,250 µM, which is in accordance with the previously reported value (Schmidt et al., 2012b An IC_{50} value of 900 µM was found for acarbose for the same method). All four compounds (**4a-d**) provided more than 50% inhibition of the enzyme at 800 µM concentrations. The compound **4c** was found to be the least active compound with 57.84% inhibition. The compounds **4b** and **4d** provided similar inhibitory patterns, with respective values of 79.73% and 73.21%. The compound **4a** was determined to be the most promising candidate for the α-glucosidase inhibition, with 96.72% inhibition.

In the case of the α-amylase enzyme, the standard acarbose provided 93.23% inhibition at 1 µM concentration. The inhibition percentages of the compounds **4a-d** ranged between 80.58%-93.05% at 800 µM, which reveals the compounds to have very low potency toward α-amylase compared to acarbose. The compounds **4c** and **4d** provided similar inhibitory patterns with respective values of 82.24% and 80.58%, while the compounds **4a** and **4b** were determined as the most potent compounds, with respective inhibition values of 93.05% and 88.29%.

Non-selective inhibition of the above-mentioned enzymes is thought to be associated with the usual side effects (e.g., abdominal pain, nausea, vomiting, meteorism, diarrhea) of the drugs in the market. α-Amylase is responsible for hydrolyzing polysaccharides such as amylose and amylopectin to oligosac-

charides such as maltose and maltotriose. It is located in saliva and pancreatic secretions. Cleaving the bonds in oligosaccharides to form monosaccharides such as glucose is the duty of α-glucosidase, which is in the small intestine. Relative greater inhibition of α-amylase compared to α-glucosidase may result in incompletely digested polysaccharides (carbohydrates) reaching the intestine and causing abnormal fermentation (Apostolidis & Lee, 2010; Beidokhti et al., 2020). All tested compounds (**4a-d**) have better inhibitory profiles than acarbose, which is more than 1,250 times more sensitive to amylase according to the current results. Compound **4a** has particularly promising results, with higher potency and selectivity compared to the standard.

The structural evaluations of the compounds revealed the presence of any substitution on the N-edge of the thiosemicarbazones to decrease the inhibition potency for both enzymes. The unsubstituted compound **4a** provided the best inhibition for α-amylase and α-glucosidase enzymes, and its inhibition potency behavior toward the designated enzymes was found to be identical to the methyl-substituted compound **4b**. Although, the ethyl-substituted counterpart **4d** showed similar inhibition values for the enzymes, a dramatic difference was obtained for the dimethyl-substituted thiosemicarbazone **4c**. The compound was a promising candidate for the α-amylase enzyme; however, its inhibition potency had decreased to 57.84% for the α-glucosidase enzyme.

Molecular docking

The computational study has been carried out to understand the behavior of the compounds on the catalytic side of the enzyme pockets. The study has evaluated interactions between the amino acid residues and the chemical fragments on the compounds and attempted to make a logical explanation of how the molecules demonstrate their inhibition potencies.

Figures 2-5 show the 2D interactions of the compounds with the α-amylase enzyme. The conventional hydrogen bonds have been identified as stronger interactions, with the number of interactions determining the compounds' inhibition potencies. Compound **4a** should importantly be noted to have also been determined as the most active candidate for the computational study, as it has the highest number of conventional hydrogen bonds. His201, Ile235, Glu233, Asp197, and Asp300 are the residues that form hydrogen bonds with the thiosemicarbazone fragments of the molecules. Compound **4b** resulted in two different hydrogen bonds through His201 and Tyr151; however, compounds **4c** and **4d** demonstrated only hydrophilic interactions (i.e., carbon-hydrogen, pi alkyl). Pi-donor and pi-sulfur interactions are the common bindings for all compounds through the His or Asp and Tyr or Trp residues.

In the case of the α-glucosidase enzyme, the interactions between the substituents and amino acid residues on the catalytic side were found to be compatible with the results obtained from the bioactivity assay (Figures 6-9). The strongest interaction was determined to have occurred for compound **4a** with Leu428 and Pro426 due to the presence of the carbazole

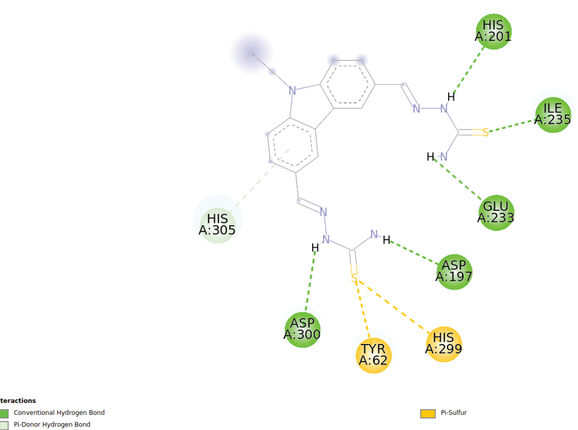

Figure 2. 2D representations of 4a to α-amylase enzyme binding site residue interactions.

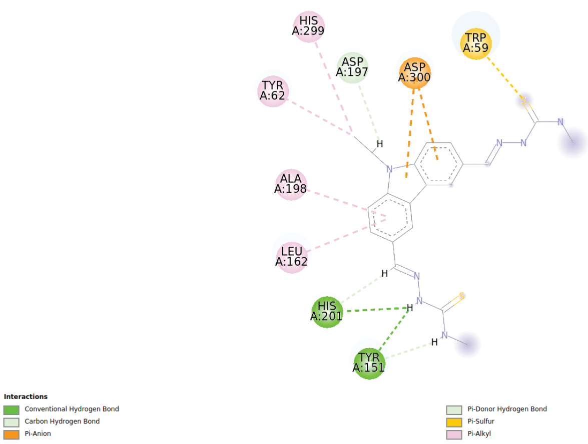

Figure 3. 2D representations of 4b to α-amylase enzyme binding site residue interactions.

Table 1: Enzyme inhibitory activity of the compounds and the standard.

Compounds (800 μM)	α-Amylase (inhibition% ± SD)	α-Glucosidase (inhibition% ± SD)
4a	93.05 ± 2.56	96.72 ± 2.66
4b	88.29 ± 0.53	79.73 ± 1.00
4c	82.24 ± 0.32	57.84 ± 1.33
4d	80.58 ± 0.96	73.21 ± 3.93
Acarbose	93.23 ± 1.26*	70.95 ± 0.46**

*C = 1 μM; **c = 1250 μM

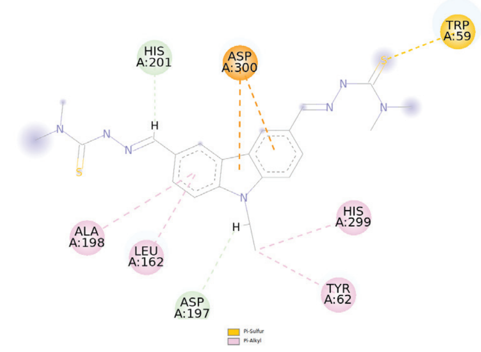

Figure 4. 2D representations of 4c to α-amylase enzyme binding site residue interactions.

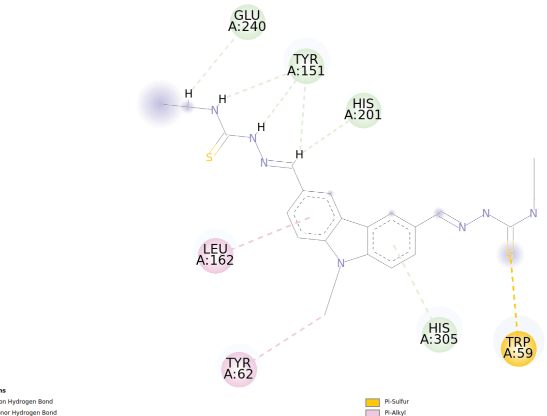

Figure 5. 2D representations of 4d to α-amylase enzyme binding site residue interactions.

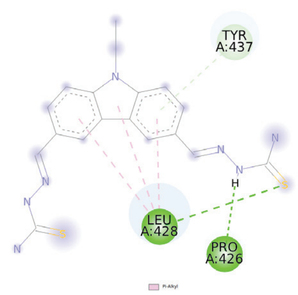

Figure 6. 2D representations of 4a to α-glucosidase enzyme binding site residue interactions.

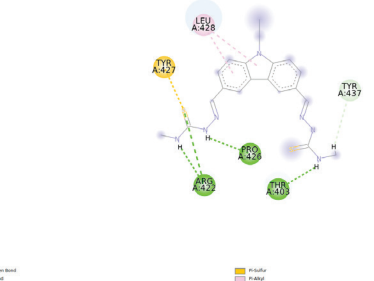

Figure 7. 2D representations of 4b to α-glucosidase enzyme binding site residue interactions.

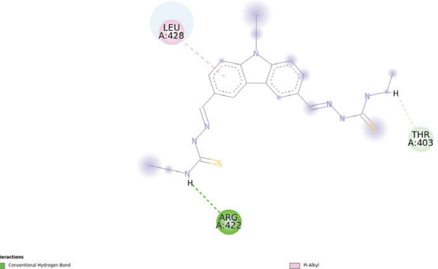

Figure 8. 2D representations of 4c to α-glucosidase enzyme binding site residue interactions.

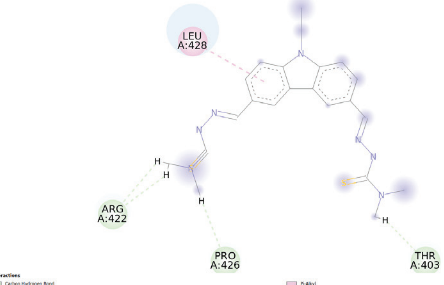

Figure 9. 2D representations of 4c to α-glucosidase enzyme binding site residue interactions.

background, sulfur atom, and amino residue on the thiosemicarbazone moiety. Similar interactions were also obtained for compound **4b** with the amino acid residues Pro426, Arg422, and Thr403. The weakest binding pattern was observed with compound **4c** due to the non-hydrogen bonding and hydrophobic interactions (i.e., C-H and pi-alkyl bonds provided the lowest potency for the α-glucosidase enzyme. As a result, compound **4d** was determined to be the third best compound and the only one with a conventional H bond to Arg422.

CONCLUSION

This study has chosen hybrid molecules derived from carbazole and thiosemicarbazones for the individually potency regarding antidiabetic study and evaluated them for their α-amylase and α-glucosidase inhibitory activity. Four compounds with different substituents on the thiosemicarbazone moiety were selected in terms of their different bonding patterns with regard to the catalytic properties of the designated enzymes. The molecular docking study was carried out to create logical explanations for the practical results obtained from the bioassays. The theoretical data obtained from the computational study is importantly noted to be compatible with the practical results. All tested compounds revealed higher potencies than acarbose in terms of α-glucosidase inhibition. Furthermore, all compounds showed very low inhibitions toward α-amylase compared to acarbose, which has been suggested for eliminating the usual side effects of acarbose. Compound **4a** was found to be the most promising candidate for both its potency and relative selectivity. The presence of an unsubstituted NH_2 functional group on the N edge of the thiosemicarbazone created more conventional H bonds with the amino acid residues and better binding ability on the catalytic side. The increased

binding ability may possibly result in better inhibition percentages for the α-amylase and α-glucosidase enzymes. The addition of any substituents on the designated area of the molecule decreased the potency due to the weaker interactions on the enzyme pockets. Overall, the carbazole-based thiosemicarbazones were found to be more potent and selective candidates for α-glucosidase enzyme inhibition.

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Original Article

Antibiotic susceptibility and biofilm formation of multi-drug resistant Gram-negative bacteria

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ABSTRACT

Background and Aims: Gram-negative bacteria are important pathogens that can cause community- and hospital-acquired infections as well as opportunistic infections, with antimicrobial resistance in Gram-negative bacteria becoming a growing crisis in clinical medicine. Biofilm formation is one of the mechanisms of bacterial resistance, which makes bacteria less susceptible to antimicrobial agents and unable to be killed by host immune mechanisms. Therefore, this study investigates the antimicrobial resistances and biofilm-forming abilities of a total of 98 Gram-negative strains isolated from various clinical specimens.

Methods: A disc diffusion assay was performed to detect the susceptibility profiles of 98 Gram-negative strains. The biofilm-forming abilities of strains were also determined using the Crystal Violet assay.

Results: Concerning the disc diffusion assay, most of the isolates were found to be resistant to carbapenems, with more than 90% of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* isolates being found resistant to ceftazidime and piperacillin. Most of the *Pseudomonas aeruginosa* isolates (75%) were found to be resistant to imipenem and aztreonam. All isolates had the ability to form biofilms. Overall, 56% of isolates were strong formers, and 29% were moderate biofilm formers. Strong biofilm formation was observed in most strains except for *K. pneumoniae*.

Conclusion: The surveillance of susceptibility profiles and biofilm formation is important for determining their variable susceptibility patterns and aiding in the appropriate management of infections caused by these organisms.

Keywords: Multi-drug resistance, antibiotic susceptibility, biofilm

INTRODUCTION

Gram-negative bacteria differ from Gram-positive bacteria in that they have a thinner peptidoglycan layer and an outer membrane that acts an important mechanical barrier. They are very common in nature and cause many serious infections (Eichenberger & Thaden, 2019). Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are some of the Gram-negative bacteria that most commonly cause nosocomial infections such as ventilator-associated pneumonia, urinary tract infections, and sepsis and that develop resistances due to the inappropriate use of antibiotics (Hammoudi & Ayoub, 2020). Antimicrobial resistance can occur through numerous mechanisms, including antibiotic degradation by enzymes, impermeability of the bacteria to the antibiotic, antibiotic target modification, genetic transfer of resistance genes, and increase in bacterial membrane permeability (Eichenberger & Thaden, 2019; Vivas, Barbosa, Dolabela, & Jain, 2019). Due to the natural structure of the outer

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membrane, Gram-negative bacteria are naturally resistant to some large-scale antibiotics such as vancomycin. In addition, modifications in the outer membrane such as changes in hydrophobic properties and porin changes in the outer membrane may cause resistance to develop. Thus, Gram-negative bacteria tend to be more resistant to antibiotics than Gram-positives (Breijyeh, Jubeh, & Karaman, 2020).

Bacteria are defined as multi-drug resistant if they are resistant to three or more antimicrobial classes (Magiorakos et al., 2012; Perdikouri et al., 2019). Multi-drug resistance is an important factor that increases the length of hospitalization stay, mortality, and cost of treatment (Peters et al., 2019; Thaden et al. 2017).

The ESKAPE group is an important group of bacteria that cause nosocomial infections and are able to avoid the effects of antibiotics with resistance mechanisms. Gram-negative bacteria form a large part of this ESKAPE group, which gets its name from the first letter of the following bacteria: *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumanni*, *P. aeruginosa*, and *Enterobacter* species (Rice,, 2008). These microorganisms are serious threats to hospitals because they can easily contaminate hospital surfaces and medical equipment. In 2019, The Centers for Disease Control and Prevention (CDC) reported carbapenem-resistant *Acinetobacter* spp. and carbapenem-resistant *Enterobacteriaceae* to be urgent threats requiring immediate and aggressive action due to their high risk of outbreaks in hospitals and nursing homes.

Multi-drug resistance is not the only cause of treatment failure; some bacteria also have the ability to form biofilms can make them up to 1,000 times more resistant to antibiotics. Biofilms are communities of microorganisms embedded in a self-produced exopolysaccharide matrix containing various substances such as polysaccharides, proteins, and DNA (Cepas et al., 2019; Wang, Zhao, Chao, Xie, & Wang, 2020). Biofilms can form on biotic and/or abiotic surfaces. The formation of biofilms on medical devices such as ventilators and implants applied externally to patients poses a serious risk in terms of hospital infections. Biofilm formation is a complex process consisting of the following many stages: attachment, micro-colony formation, maturation and formation of the architecture of the biofilm, and detachment (Jamal et al., 2018). Biofilm forming ability causes bacteria to become more resistant to antibiotics and bodily defense mechanisms. Gram-negative bacteria with acquired antimicrobial resistance and biofilm-forming ability are a very serious threat causing nosocomial infections. This study thus aims to evaluate the antimicrobial resistances and biofilm formations of a total of 98 Gram-negative strains.

MATERIALS AND METHODS

Strains

A total of 98 Gram-negative bacteria, including 37 *Acinetobacter baumannii*, 34 *Klebsiella pneumoniae*, 16 *Pseudomonas aeruginosa*, 5 *Escherichia coli*, 4 *Stenotrophomonas maltophilia*, 1 *Serratia sp.*, and 1 *Enterobacter sp.* isolates from various specimens including blood, urine, sputum, abscess, tracheal aspirate, and bronchoalveolar lavage fluid were obtained from the Synevo Laboratories Ankara Central Laboratory in Turkey (2020-2021). Identification of the strains was performed using the Vitek 2 (BioMerieux, France), API 20 E, and API 20 NE (BioMerieux, France) systems. Before the analyses, each isolate was cultured on tryptic soy agar (TSA, Difco Sparks, MD, USA) plates to ensure viability.

Disc diffusion assay

Imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ceftazidime (30 µg), cefepime (30 µg), piperacillin (100 µg), and aztreonam (30 µg) discs (Bioanalyse, Turkey) were used for the antibiotic susceptibility testing. The antimicrobial susceptibility assay was performed using the disc diffusion method on Mueller-Hinton agar (MHA-Sigma-Aldrich, St. Louis, MO, USA) in accordance with the Clinical and Laboratory Standards Institute (CLSI) M100-Ed 31 (CLSI, 2021). Bacterial suspensions were prepared by selecting similar colonies from an overnight culture with a sterile loop and suspending the colonies in sterile saline (0.85% NaCl w/v in water) at the density of a McFarland 0.5 standard, approximately corresponding to $1–2 \times 10^8$ cfu/ml. Suspensions were swabbed on MHA (9 cm plates, with 25 ml medium). The plates were air dried for 15–20 min, and filter paper discs (6 mm diameter; Bioanalyse, Turkey) containing antibiotics were placed onto the inoculated agar. The plates were incubated at 37 °C for 24 h. The next day, the inhibition zone diameters were measured in millimeters and evaluated according to the CLSI breakpoint tables.

Biofilm formation

The biofilm forming abilities of the isolates were investigated using the Crystal Violet (CV) staining method (Dosler, & Karaaslan, 2014; Peeters, Nelis, & Coenye, 2008). Bacteria were adjusted with tryptic soy broth (TSB) glucose to a final concentration of approximately 1×10^7 cfu/ml. For the biofilm formation, the cell suspensions were placed into the wells of the microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubated for 24 h at 37°C. The next day, the remaining medium was aspirated gently, and the non-adherent cells were removed. For the biofilm fixations, 100 µl 99% of methanol was added to the wells, left to wait for 15 min, and aspirated; then the plates were air-dried. Next, the wells were stained with 100 µl 0.1% CV for 5 min, after which the excess CV was removed by washing the plates with tap water. The bound CV was solubilized by adding 95% ethanol over 30 min. Optical density (OD) was measured at 600 nm. For each isolate, biofilm formation was measured in triplicate, and *P. aeruginosa* PAO1 was used as a strong biofilm producer. Biofilm formation (weak, moderate, and strong) was interpreted as follows.

OD (isolate) ≤ OD (negative control) = negative biofilm formation;

OD (negative control) ≤ OD (isolate) ≤ 2xOD (negative control) = weak biofilm formation;

2xOD (negative control) ≤ OD (isolate) ≤ 4xOD (negative control) = moderate biofilm formation;

4xOD (negative control) ≤ OD (isolate) = Strong biofilm formation (Nirwati et al., 2019).

RESULTS

Disc diffusion assay

Table 1 summarizes the antibiotic disc diffusion susceptibility results, according to which most of the isolates were found resistant to carbapenems. More than 90% of the *A. baumannii*, *K. pneumoniae*, and *E. coli* isolates were found to be resistant to ceftazidime and piperacillin. Ciprofloxacin and piperacillin were ineffective against *E. coli*. Most of the *P. aeruginosa* isolates (75%) were found to be resistant to imipenem and aztreonam. Apart from aztreonam against *Serratia* sp. and gentamicin and amikacin against *Enterobacter* sp., all antibiotics were found ineffective.

Biofilm formation assay

All the isolates demonstrated the ability to form biofilms. Strong biofilm formation was observed in most of the strains except *K. pneumoniae*. Weak biofilm formation was only observed in five strains of *A. baumannii* (13.51%), nine strains of *K. pneumoniae* (26.47%), and one strain of *S. maltophilia* (25%; Table 2).

DISCUSSION

Gram-negative bacteria, most commonly *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, and *Enterobacter spp.* are responsible for causing various diseases, including bloodstream infections, urinary tract infections, pneumonia, wound or surgical site infections, and nosocomial infections (Dumaru, Baral, R., & Shrestha, et al., 2019). Antimicrobial resistance for Gram-negative bacteria is a growing global health threat. The World Health Organization (WHO) published a list of bacteria (grouped by priority as critical, high, and medium) that urgently need novel antibiotics in order to be fought, with most of these being Gram-negative bacteria (Breijyeh, Jubeh, & Karaman, 2020).

This study has investigated the antimicrobial resistance patterns of 98 Gram-negative bacteria. According to the results, most of the *A. baumanni* isolates were found to be resistant to all the studied antibiotics, even carbapenems. Carbapenems are one of the most-used and effective antibiotics against Gram-negative bacteria due to their broad-spectrum antibacterial activity that targets penicillin-binding proteins while

Table 1. Antibiotic resistance patterns of bacteria.

	A. baumannii (n=37)	*K. pneumoniae* (n=34)	*P. aeruginosa* (n=16)	*E. coli* (n=5)	*S. maltophilia* (n=4)	*Serratia sp.* (n=1)	*Enterobacter* sp. (n=1)
IPM	81	29	75	60	*	100	100
MEM	89	79	68	40	*	100	100
GN	91	79	56	20	*	100	0
TM	78	82	50	60	*	100	100
AN	91	76	50	20	*	100	0
CIP	94	85	56	100	*	100	100
LVX	83	85	50	100	50	100	100
CAZ	91	97	50	100	*	100	100
FEP	91	85	37	100	*	100	100
PIP	97	94	50	100	*	100	100
ATM	*	88	75	100	*	0	100

* Antibiotics not tested/not recommended by CLSI
Imipenem(IPM), meropenem (MEM), gentamicin (GN), tobramycin (TM), amikacin (AN), ciprofloxacin (CIP), levofloxacin (LVX), ceftazidime (CAZ), cefepime (FEP), piperacillin (PIP) and aztreonam (ATM)

Table 2. Biofilm formation rates of isolates.

	Strong	Moderate	Weak
A. baumannii (n=37)	65% (n=24)	22% (n=8)	13% (n=5)
K. pneumoniae (n=34)	29% (n=10)	44% (n=15)	26% (n=9)
P. aeruginosa (n=16)	81% (n=13)	18% (n=3)	-
E. coli (n=5)	60% (n=3)	40% (n=2)	-
S. maltophilia (n=4)	75% (n=3)	-	25% (n=1)
Serratia sp. (n=1)	100% (n=1)	-	-
Enterobacter sp. (n=1)	100% (n=1)	-	-
Total (n=98)	56% (n=55)	28% (n=28)	15% (n=15)

being relatively resistant to hydrolysis by most β-lactamases (El-Gamal et al., 2017). Infections caused by the multidrug-resistant *A. baumannii* strains are one of the most troublesome infections to treat because most clinical strains resistant to carbapenems are generally resistant to all classes of β-lactams as well as other classes of antibiotics (Malone & Kwon, 2013). Resistance rates are increasing all over the world, with 40%-70% of the strains being shown to be carbapenem resistant in infections acquired in intensive care units. The pattern of antibiotic resistance for *A. baumannii* in this study is similar to those found in many previous studies (Namaei et al., 2021, Yadav, Bhujel, & Mishra, 2020).

K. pneumoniae is another major threat to public health and the most common cause of hospital- and community-acquired infections, with carbapenem-resistant strains having been reported recently (Candan & Aksöz 2015). Among the antibiotics tested in this study, 71% of *K. pneumoniae* strains were susceptible to imipenem, while other antibiotics were mostly ineffective. Although some studies reported a lower resistance rate in *K. pneumoniae* against antibiotics (Dumaru et al., 2019; Cepas et al. 2019), resistance has gradually been increasing globally, similar to what this study found.

P. aeruginosa is an important pathogen that is able to cause bloodstream infections, surgical site infections, and lower respiratory system infections, especially in cystic fibrosis and immunocompromised patients. It is resistant to a variety of antibiotics, including carbapenems, aminoglycosides, quinolones, and β-lactams (Pang, Raudonis, Glick, Lin, & Cheng, 2019). Most of the *P. aeruginosa* isolates in this study were found to be resistant to carbapenems and aztreonam, with 37% of them being susceptible to cefepime. Cefepime is a fourth-generation cephalosporin and shows bactericidal activity by binding to penicillin-binding proteins and inhibiting peptidoglycan synthesis. Cefepime is widely used for the treatment of moderate-to-severe infections, including infections caused by *P. aeruginosa* (Jia et al., 2020). A similar pattern of cefepime resistance was also reported by Dumaru et al. (2019) for *P. aeruginosa*.

The current study found ciprofloxacin and piperacillin to be ineffective against the *E. coli* isolates, with all the *E. coli* strains also being found resistant to levofloxacin, ceftazidime, cefepime, and aztreonam. Only one *E. coli* isolate was found to be resistant to amikacin and gentamicin. Despite not being widely used due to concerns of toxicity, aminoglycosides including amikacin and gentamicin are still important therapeutic options for treating serious infections caused by Gram-negative bacteria (Bader, Loeb, Leto, & Brooks, 2020). A few recent studies in Turkiye on *E. coli* have shown amikacin and gentamicin to be effective against it, similar to this study's results (Mirza & Sancak 2020; Avcıoğlu & Behçet, 2020; İnce et al., 2021).

S. maltophilia is an important pathogen that primarily causes respiratory tract infections such as pneumonia and acute exacerbations of chronic obstructive pulmonary disease. The antibiotic options for treating *S. maltophilia* infections are limited due to its intrinsic resistance to a wide variety of antibiotics, including aminoglycosides, most β-lactams, and tetracyclines (Mojica et al., 2022). According to the current study's results, two of the four isolates were found to be susceptible to levofloxacin. Chung et al. (2012) studied the antimicrobial susceptibility of 90 clinical isolates of *S. maltophilia* and also showed levofloxacin to be effective against most of the isolates tested.

Despite the limited number of *Serratia sp.* and *Enterobacter sp.* strains studied here, aztreonam was found to be effective against *Serratia sp.* and amikacin against *Enterobacter sp.*

According to the National Institutes of Health, biofilms are a complex structure comprising microbial cells and extracellular matrix and are estimated to be responsible for 65% of all microbial infections and 80% of chronic infections (Jamal et al., 2018). Biofilm infections include non-device and device-associated infections such as central venous catheters, breast implants, urinary catheters, mechanical heart valves, peritoneal dialysis catheters, ventricular shunts, prosthetic joints, and contact lenses (Jamal et al., 2018).

Overall, 56.12% of the isolates were detected as strong and 28.57% as moderate biofilm formers in this study. These results are consistent with those from Dumaru et al.'s (2019) study, who detected 62.73% of the Gram-negative isolates, which included *E. coli*, *Acinetobacter sp.*, *Klebsiella sp.*, and *Pseudomonas* sp., to be biofilm positive. Similarly, Cepas et al. (2018) found a total of 49.3% of the *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates to have the ability to form biofilms at respective rates of 30.3%, 37.6%, and 76.5%.

Biofilm formation is known to represent a conserved growth mode that makes bacteria less susceptible to antibiotics and unable to be killed by host immune mechanisms (Del Pozo, 2018). Antibiotic treatments may not be effective once the biofilm has matured (Jamal et al., 2018), so the high rate of biofilm formation in our strains may result in antibiotic resistance in addition to treatment failure.

CONCLUSION

To conclude, Gram-negative bacteria, most commonly *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*, are mostly multi-drug resistant and becoming increasingly resistant to all available antibiotics. Antimicrobial resistance is a significant global problem, and WHO has declared it to be one of the top 10 global public health threats facing humanity. Therefore, surveilling Gram-negative bacteria's susceptibility profiles and biofilm formation is important because knowing the variable susceptibility patterns can aid in the appropriate management of the infections they cause.

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Original Article

Anticholinesterase and antityrosinase activities of endemic *Prangos heyniae* H. Duman & M. F. Watson and its metabolites

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ABSTRACT

Background and Aims: *Prangos* Lindl. (Apiaceae) are abundant in coumarins. Previously, along with *n*-hexane(HEX), chloroform($CHCl_3$), and methanol(MeOH) extracts, 8 molecules named osthol(**1**), isoimperatorin(**2**), oxypeucedanin(**3**), 7-methoxy-isoarnottinin-4'-O-β-ᴅ-glucopyranoside(**4**), 7-methoxy-isoarnottinin-4'-O-rutinoside(**5**), oxypeucedanin hydrate-3'-O-β-ᴅ-glucopyranoside(**6**), 1-methylethyl-6-O-D-apio-β-ᴅ-furanosyl-β-ᴅ-glucopyranoside(**7**), and cnidioside A(**8**) were obtained from the roots of endemic *Prangos heyniae* H. Duman & M. F. Watson. **4** and **5** were reported as novel compounds. Coumarins are known for their neuroprotective properties. Tyrosinase and cholinesterase enzymes play a key role in the course of neurodegenerative diseases such as Parkinson's and Alzheimer's disease(AD), respectively. Therefore, we aimed to evaluate the antityrosinase and anticholinesterase effects of the extracts and compounds **1-8** from *Prangos heyniae* roots.

Methods: Tyrosinase and acetylcholinesterase-butyrylcholinesterase(AChE-BChE) inhibitory activities of the samples were evaluated spectrophotometrically. The screening of the samples was carried on at 1000 µg/mL. Results of triplicate analyses of the samples were given as IC_{50} values obtained through linear regression analysis. Kojic acid and galantamine were used as positive controls for antityrosinase and anticholinesterase experiments, respectively.

Results: Only MeOH extract showed antityrosinase activity with an IC_{50} value of 543.37±7.45 µg/mL. $CHCl_3$ extract exhibited both AChE and BChE inhibitory activities with IC_{50} values of 273.92 ± 32.07 and 38.68±2.56 µg/mL, respectively. Among tested compounds, **6** showed a weak BChE-specific inhibitory activity (IC_{50}= 91.93±3.86µg/mL) and managed to possess 40 times inferior activity than galantamine(IC_{50}= 2.25 ± 0.05µg/mL).

Conclusion: The $CHCl_3$ extract displayed a good BChE inhibitory activity. These findings suggested that *Prangos heyniae* could be a valuable natural source to develop novel BChE inhibitors with further studies against AD.

Keywords: *Prangos heyniae*, coumarin, anticholinesterase activity, antityrosinase activity, oxypeucedanin hydrate-3'-O-β-ᴅ-glucopyranoside

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INTRODUCTION

Prangos Lindl. (Apiaceae) genus is an Iran-Turan element and is represented by 45 species worldwide (Lyskov, Degtjareva, Samigullin, & Pimenov, 2017). The genus is spread out from Europe to Tibet, mostly growing in Turkey and Iran (Lyskov et al., 2017; Menemen, 2012; Mottaghipisheh, Kiss, Tóth, & Csupor, 2020). Species of the genus grow on calcareous rocks, basalt rocky soils, salty soils, and mountain slopes (Aytaç & Duman, 2016; B. Başer & Pehlivan, 2015; Lyskov et al., 2017; Menemen, 2012; Mottaghipisheh et al., 2020). There are 19 taxa of which 11 are endemic to Turkey (Aytaç & Duman, 2016; Behçet, Yapar, & Olgun, 2019; Menemen, 2012). Several traditional usages of the plants of the genus have been reported. In Anatolian folk medicine, the roots of the plant are beneficial as an anti-hemorrhoidal, wound-healing agent, and aphrodisiac whereas the aerial parts are used as a stimulant and carminative (Bulut, Tuzlacı, Doğan, & Şenkardes, 2014). In literature, bioactivity studies were found to be generally associated with the antibacterial, cytotoxic and antioxidant effects of *Prangos* species (Farooq et al., 2014; Kogure et al., 2004; Massumi, Fazeli, Alavi, & Ajani, 2007; Özek et al., 2007; Ulubelen et al., 1995; Zahri, Razavi, Niri, & Mohammadi, 2009). Besides, anti-inflammatory, wound healing, antiviral, hepatoprotective, antidiabetic, and vascular reactivity studies were also reported by previous studies (Doković et al., 2004; Farkhad, Farokhi, & Tukmacki, 2012; Farokhi, Farkhad, & Togmechi, 2012; Sevin et al., 2022; Shokoohinia, Sajjadi, Gholamzadeh, Fattahi, & Behbahani, 2014; Tada et al., 2002; Zahri et al., 2009). In addition to various bioactivity studies of the genus, anticholinesterase activities of the different *Prangos* species have also been investigated (Albayrak, Demir, Koyu, & Baykan, 2022; Dall'Acqua et al., 2022; Mottaghipisheh et al., 2020; Zengin et al., 2022). *Prangos heyniae* H. Duman & M. F. Watson, known as "Boz çakşır" in Anatolia, is a perennial herb and an endemic species distributed in Konya province, Turkey (Duman & Watson, 1999; Menemen, 2012). There are few studies reported on this endemic plant. Antioxidant, mosquitocidal, and anticandidal activities are the bioactivity studies conducted with *P. heyniae* (Ahmed, Güvenç, Küçükboyacı, Baldemir, & Coşkun, 2011; Öke-Altuntaş, Aslım, Duman, Gülpınar, & Kartal, 2015; Özek et al., 2018). The essential oil composition of fruits and roots of the plant have been investigated and elemol, α-pinene, , kessane and germacrene D were found as major compounds (K. H. C. Başer, Özek, Demirci, & Duman, 2000; Karahisar, Köse, İşcan, Kürkçüoğlu, & Tugay, 2022; Özek et al., 2018; Zengin et al., 2022). In addition, a sesquiterpene ketone; 3,7(11)-eudesmadien-2-one was obtained from the essential oils of *P. heyniae* fruits using preparative gas chromatography (Özek et al., 2018). The essential oils and extracts obtained from the aerial parts of *P. heyniae* have been evaluated for their antityrosinase and anticholinesterase activities in different studies (Dall'Acqua et al., 2022; Zengin et al., 2022). However, to the best of our knowledge, the plant roots have not been investigated for anticholinesterase and antityrosinase properties before. Coumarins and furanocoumarins represent the major group among the metabolites occurring in the genus *Prangos* and they are known for acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity studies related to neurodegenerative diseases (De Souza, Renn O B, & Figueroa-Villar, 2016; Mottaghipisheh et al., 2020). Tyrosinase plays an important role in melanin biosynthesis and the overproduction of melanin results in hyperpigmentation. Melanin pigment is also found in the brain, as neuromelanin, and high levels of neuromelanin have been associated with dopamine neurotoxicity, which leads to Parkinson's disease. Tyrosinase enzyme is an important target to decrease the melanocyte function, and there are several studies evaluating the tyrosinase inhibitory activity of these metabolites (Chang, 2009; Fais et al., 2009; Shu et al., 2020; Zolghadri et al., 2019). Among these compounds, osthole, oxypeucedanin, oxypeucedanin hydrate, psoralen, xanthotoxin, marmesin, heraclenin, heraclenol, imperatorin, and isoimperatorin draw attention as the main coumarins and furanocoumarins isolated from *Prangos* species (Abbas-Mohammadi et al., 2018; Bruno et al., 2021; Mottaghipisheh et al., 2020; Zengin et al., 2020). Recent studies have shown that coumarins and furanocoumarins are prominent in the search for the treatment of neurodegenerative disease. The neuroprotective activity along with mechanism studies of coumarin and furanocoumarin derivatives are increasingly supported by not only cholinergic pathway studies (Karakaya et al., 2020; Orhan et al., 2021) but also anti-amyloidogenic activity studies (Palmioli et al., 2019). Considering this information, the aim of this study was to evaluate the *in vitro* anticholinesterase and antityrosinase potential of HEX, $CHCl_3$, and MeOH extracts of the plant roots along with the compounds [Figure 1; osthol (**1**), isoimperatorin (**2**), oxypeucedanin (**3**), 7-methoxy isoarnottinin 4'-O-β-D-glucopyranoside (**4**), 7-methoxy isoarnottinin 4'-O-rutinoside (**5**), oxypeucedanin hydrate-3'-O-β-D-glucopyranoside (**6**), 1-methylethyl 6-O-D-apio-β-D-furanosyl-β-D-glucopyranoside (**7**), and cnidioside A (**8**)] obtained from our previous study (Albayrak, Demir, Kose, & Baykan, 2021). In summary, hoping to reveal the pharmacological value of this endemic plant of Turkey, our purpose is to discover the neuroprotective and anti-hyperpigmentation potential of this plant and its chemical components that could lead to the development of novel candidate metabolites for the prevention and treatment of neurodegenerative and hyperpigmentation disorders.

MATERIALS AND METHODS

Plant extracts and isolated compounds

Prangos heyniae H. Duman & M. F. Watson was collected from Konya province in 2016, identified by Prof. Dr. Serdar Gokhan Senol, Department of Biology, Faculty of Science, Ege University. A voucher specimen (IZEF-6051) was stored in the Herbarium of Ege University, Faculty of Pharmacy, Izmir, Turkey. In our previous study, air-dried roots of the plant were extracted with HEX, $CHCl_3$, and MeOH, sequentially. Moreover, 8 compounds [6 coumarin derivatives (**1**-**6**), one isopropyl glycoside (**7**), and one benzofuran derivative (**8**)] were obtained from the extracts by using chromatographic methods and were identified using spectroscopic techniques (Albayrak et al., 2021). The HEX, $CHCl_3$, and MeOH extracts and isolated molecules named osthol (**1**), isoimperatorin (**2**), oxypeucedanin (**3**), 7-methoxy isoarnottinin 4'-O-β-D-glucopyranoside (**4**), 7-methoxy isoarnottinin 4'-O-rutinoside (**5**), oxypeucedanin hydrate-3'-O-β-D-glucopyranoside (**6**), 1-methylethyl 6-O-D-apio-β-D-furanosyl-β-D-glucopyranoside (**7**), and cnidioside A (**8**) used in this study were isolated from the roots of *Prangos heyniae* in the above-mentioned work (Albayrak et al., 2021).

Figure 1. The tested compounds **1-8** from *Prangos heyniae*.

Anticholinesterase activity

AChE and BChE inhibitory activities of the extracts and isolated compounds were evaluated using a modified Ellman's method (Ellman, Courtney, Andres, & Featherstone, 1961). Electric eel AChE and horse serum BChE were used as the enzymes. Acetylthiocholine iodide and butyrylthiocholine iodide (3 mM) were selected as substrates for the enzymatic reaction, respectively. 5, 5'-Dithio-bis 2-nitrobenzoic acid (DTNB) was used for the measurement of the anticholinesterase activity. Briefly, 150 µL 0.1 M sodium phosphate buffer, DTNB (0.01 M), sample solution (prepared with DMSO: H_2O, 1:9), and acetylcholinesterase/butyrylcholinesterase (0.1 Unit/mL) enzymes were added in a 96-well microplate. Samples were pre-incubated at room temperature for 5 min at 300 rpm on an orbital shaker. The reaction was then initiated by adding 3 mM substrate (in buffer). Kinetic absorbance was measured at room temperature per 30-sec intervals through an incubation period of 10 min at 412 nm with a microplate reader. Enzyme activity was calculated with the linear change of absorbance during the incubation period compared to that of the assay using a buffer without any inhibitor. The activity results for cholinesterase inhibition were calculated by the following formula;

$$\% \text{ inhibition} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance with sodium phosphate buffer (instead of the tested sample) and A_{sample} is the absorbance with the extract/isolated compound/positive control at 412 nm. The positive control was galantamine as the reference drug. In order to evaluate the validity of our study protocol, Sigma Plot 12.0 Enzyme Kinetics Module 1.3 was used to establish the inhibition kinetics of galantamine with Lineweaver-Burk, Michaelis-Menten, and Eadie-Hofstee methods (Albayrak et al., 2022). The results of triplicate experiments were depicted with IC_{50} values (concentration that inhibits 50% of enzyme activity) obtained by linear regression analysis where 1000 µg/mL was the initial test concentration for the tested samples. The selected test samples were evaluated at concentrations of 1000 µg/mL and 15.625 µg/mL to determine their IC_{50} values. Development and optimization of the activity study protocol were done through an inhibition kinetic study with galantamine as a reference drug for ChE inhibition, for the accurate determination of enzyme inhibition assay parameters and activity results. Inhibition kinetics were analyzed with acetylthiocholine iodide as a single substrate within the concentration range of 31.25–125 µM and galantamine as a single inhibitor within the range of 0.0875–0.35 µM with a blank (0 µM). The determined competitive type inhibition correlated with the literature for galantamine

thus approving the validity of our developed study protocol (Albayrak et al., 2022; Ellman et al., 1961).

Antityrosinase activity

An adapted 96-well microplate assay was conducted for the determination of tyrosinase inhibitory activity. Kojic acid was used as the reference drug. Twenty-five µl of samples (in pH 6.8 phosphate buffer) were mixed with 150 µl 2 mM L-dopa (in pH 6.8 phosphate buffer). Then, this mixture was pre-incubated at 25 °C in the dark for 2 min. Finally, 25 µl of tyrosinase enzyme (50 Unit/mL in phosphate buffer) was added and the whole mixture was incubated for 10 min at 25 °C. Kinetic readings at 30-sec intervals were recorded with a microplate reader (Clariostar, BMG Labtech) at 475 nm to determine the linear change of the absorbance during dopachrome formation within the 10 min reaction period. Tyrosinase inhibitory activity results were calculated with the following formula;

$$\% \text{ inhibition} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance with the sample solvent (DMSO: water 2:3) and A_{sample} is the absorbance with the extract/isolated compound at 475 nm. The positive control was kojic acid. The results of the triplicate analysis were depicted as IC_{50} values obtained through linear regression analysis where 1000 µg/mL was the initial test concentration for the tested samples. The selected test samples were evaluated at concentrations of 1000 µg/mL and 15.625 µg/mL to determine their IC_{50} values. (Koyu, Kazan, Demir, Haznedaroglu, & Yesil-Celiktas, 2018).

Statistical analysis

The data were analyzed using GraphPad Prism version 5.03 program (GraphPad Software, San Diego California, USA). The data were given as '± standard deviation of the mean'. The results were given as IC_{50} values of tested samples from triplicate analysis. The level of significance was set as $p < 0.05$.

Table 1. Anticholinesterase and antityrosinase activities of *P. heyniae* extracts and compounds 1-8.

Extracts/ Compounds	IC_{50} ± S.D. [µg/mL] [a] AChE	BChE	Tyr
HEX	-	-	-
$CHCl_3$	273.92 ± 32.07 (R^2=0.9724)	38.68 ± 2.56 (R^2=0.9717)	-
MeOH	-	-	543.37 ± 7.45 (R^2=0.9654)
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	-	91.93 ± 3.86 (R^2=0.9959)	-
7	-	-	-
8	-	-	-
Galantamine [b]	0.22 ± 0.02 (R^2=0.9571)	2.25 ± 0.05 (R^2=0.9518)	
Kojic acid [c]			3.38 ± 0.17 (R^2=0.9502)

[a] Values are means of three independent samples with triplicate determination. The IC_{50} is represented as mean±standard deviation obtained from dose-response curves by linear regression. Units of concentrations are expressed as [µg/mL]

[b] Standard for cholinesterase inhibitory activities.

[c] Standard for tyrosinase inhibitory activities.

Dash means no activity at 1000 µg/mL.

RESULTS AND DISCUSSION

Anticholinesterase activity

HEX, $CHCl_3$, and MeOH extracts of *P. heyniae* roots, and 8 pure compounds (Figure 1) were evaluated for their AChE and BChE inhibitory activities. IC_{50} values of the tested extracts are given in Table 1. Linearity was determined with R^2 values as above 0.9500 and relative standard deviation values (RSD%) were determined as below 11.71% for the extracts and 4.2% for the compounds. The HEX and MeOH extracts did not show AChE inhibitory activity at the concentration of 1000 µg/mL. However, $CHCl_3$ extract inhibited both AChE and BChE enzymes with IC_{50} values of 273.92 ± 32.07 µg/mL and 38.68 ± 2.56 µg/mL, respectively. It exhibited weak AChE inhibitory activity but showed good BChE inhibitory activity (17-fold inferior activity than galantamine). Extracts and essential oils obtained from different parts of *Prangos* species have previously been evaluated for their anticholinesterase activities to discover potential sources of neuroprotective agents (Abbas-Mohammadi et al., 2018; Bruno et al., 2021; Dall'Acqua et al., 2022; Zengin et al., 2022, 2020). In a previous study, essential oils, HEX, dichloromethane (DCM) and MeOH extracts from the aerial parts of *P. gaube* were tested for their anticholinesterase activities and among the tested materials, DCM extract (3.51±0.24 mg GEs/g, galantamine equivalents) was reported as the most potent BChE inhibitor (Bahadori, Zengin, Bahadori, Maggi, & Dinparast, 2017). In another study, HEX, ethyl acetate (EtOAc) and MeOH extracts from the aerial parts of *P. ferulacea* were investigated for AChE inhibitory activity where HEX (200 µg/mL) and EtOAc extracts (200 µg/mL) were the most effective extracts and showed inhibition of 75.6±2.8% and 63.8±1.3%, respectively (Abbas-Mohammadi et al., 2018). Coumarins were indicated as the main group responsible for high activity in both studies (Abbas-Mohammadi et al., 2018; Bahadori et al., 2017). Recently, Zengin et al. investigated the essential oils from the aerial parts of *P. uechtritzii*, *P. meliocarpoides* var. *meliocarpoides,* and *P. heyniae* for their cholinesterase inhibitory properties and among the tested samples, only the essential oil of *P. heyniae* (9.85±0.20 mg GALAE/g, galantamine equivalents) showed BChE specific inhibitory activity (Zengin et al., 2022). Dall'Acqua et al. investigated the HEX, EtOAc, MeOH, and water extracts from the aerial parts of *P. uechtritzii*, *P. meliocarpoides* var. *meliocarpoides,* and *P. heyniae* for their cholinesterase inhibitory properties. HEX (AChE=2.39±00.6 mg GALAE/g, BChE=7.83±0.18 mg GALAE/g, galantamine equivalents) and

EtOAc (AChE=1.58±0.38 mg GALAE/g, BChE=7.64±0.15 mg GALAE/g, galantamine equivalents) extracts of *P. heyniae* displayed BChE-specific inhibitory activities. Coumarins and hydrolyzable tannins were reported as responsible compounds for the activity (Dall'Acqua et al., 2022). Considering the studies with *Prangos* species on cholinesterase inhibition along with our results, *P. heyniae* can be considered as a candidate source for neurotherapeutic agents, especially as a potential BChE inhibitor drug source. Among the tested isolated compounds, **6** showed BChE inhibitory activity with an IC_{50} value of 91.93 ± 3.86 µg/mL. It demonstrated 40 times inferior activity than galantamine (IC_{50}= 2.25 ± 0.05 µg/mL). However, it did not inhibit AChE at 1000 µg/mL. In previous studies, oxypeucedanin hydrate, the aglycone moiety of **6,** was inactive against AChE and BChE enzymes *in vitro* (Albayrak et al., 2022; Orhan et al., 2021; Youkwan, Sutthivaiyakit, & Sutthivaiyakit, 2010). The enhanced effect of **6** on BChE comparing its aglycone could be relevant to the presence and position of the glucose moiety at C-3'. In studies against AD, AChE seems to have a more active role than BChE in regulating acetylcholine levels in the healthy brain or early stages of AD. However, AChE activity decreases with acetylcholine in the mid to late stages of the disease, while BChE activity continues to increase (Greig, Lahiri, & Sambamurti, 2002; Greig et al., 2001). Hence, BChE could also be a significant contributor to the decrease of acetylcholine levels in AD (Walsh, Rockwood, Martin, & Darvesh, 2011). Therefore, the inhibition of the BChE is as important as the inhibition of AChE. In many studies, coumarins and furanocoumarins have been found to exhibit selectivity for BChE (De Souza et al., 2016). Especially, the presence of the prenyl moiety at the C-8 position (Granica et al., 2013; Wszelaki, Paradowska, Jamróz, Granica, & Kiss, 2011), and the presence of the furan ring at the C-6 position (Özbek et al., 2018; So & Young, 2007) were related with the increase of BChE inhibitory activity of coumarins and furanocoumarins and these studies correlate with our findings for the BChE inhibitory effect of oxypeucedanin hydrate-3'-O-β-ᴅ-glucopyranoside (**6**). The other compounds did not show AChE and BChE inhibitory activity at 1000 µg/mL. The AChE and BChE inhibitory activities of CHCl3 extract are more potent than the inhibitory activities of the isolated compounds **1**-**8**. In the BChE inhibition experiment, the extract exhibited 2.4 times (IC_{50}=38.68±2.56 µg/mL) inferior activity than galantamine (IC_{50}=2.25±0.05µg/mL), while compound **6** (IC_{50}=91.93±3.86µg/mL) showed 40 times inferior activity than the reference. The reason for the extract's superior activity could be due to the synergistic effect of all the molecules or due to other active non-isolated compounds in the extract. However, further studies are needed to reveal the activity mechanism of the extract and the compounds.

Antityrosinase activity

All three extracts along with the pure compounds (Figure 1) were tested for their antityrosinase activity. IC_{50} values of the tested extracts are given in Table 1. Linearity was determined with R^2 values above 0.9500 and relative standard deviation values (RSD%) were determined as below 1.37% for all analyzed samples. Only MeOH extract inhibited tyrosinase with an IC_{50} value of 543.37 ± 7.45 µg/mL. The extract exhibited 160-fold inferior activity than the reference drug, kojic acid (IC_{50}= 3.38 ± 0.17 µg/mL). Compounds **1**-**8** did not show any effect against the enzyme at 1000 µg/mL. There are few tyrosinase inhibitory studies with *Prangos* species (Bahadori et al., 2017; Dall'Acqua et al., 2022; Orhan et al., 2021; Zengin et al., 2022, 2020). Recently, Zengin et al. investigated the essential oils from the aerial parts of *P. uechtritzii*, *P. meliocarpoides* var. *meliocarpoides,* and *P. heyniae* for their tyrosinase inhibitory properties and among the tested samples, the essential oils of *P. meliocarpoides* var. *meliocarpoides* (69.56±4.80 mg KAE/g, kojic acid equivalents) were reported to display the strongest tyrosinase inhibitory activity. In that study, *P. heyniae* (53.91±2.11 mg KAE/g, kojic acid equivalents) was indicated to possess moderate antityrosinase activity (Zengin et al., 2022). Dall'Acqua et al. investigated the HEX, EtOAc, MeOH and water extracts from the aerial parts of *P. uechtritzii*, *P. meliocarpoides* var. *meliocarpoides,* and *P. heyniae* for their tyrosinase inhibitory properties and among the tested samples, the HEX extract of *P. meliocarpoides* var. *meliocarpoides* (81.15±0.19 mg KAE/g, kojic acid equivalents) was reported to display the strongest tyrosinase inhibitory activity. In the same study, *P. heyniae* displayed the lowest activity among the three species. The MeOH extract of *P. heyniae* (65.20±0.89 mg KAE/g, kojic acid equivalents) was indicated to possess the strongest antityrosinase activity among the other extracts and conformed with our study (Dall'Acqua et al., 2022). This can be attributed to the polar compounds such as glycosylated coumarins and tannins in the plant. In another study, 17 coumarin derivatives including osthol, isoimperatorin, oxypeucedanin and oxypeucedanin hydrate (the aglycone unit of compound **6**) were evaluated, and none of the tested compounds showed anti-tyrosinase activity at 100 µg/mL (Orhan et al., 2021). *Prangos* species and coumarins seem to show weak to moderate antityrosinase activity in previous studies, and our results were consistent with the literature (Dall'Acqua et al., 2022; Erdogan Orhan, Orhan, & Gurkas, 2011; Shu et al., 2020; Zengin et al., 2022).

CONCLUSION

In conclusion, HEX, $CHCl_3$, and MeOH extracts of the roots of endemic *P. heyniae* and the isolated metabolites; 7-methoxy isoarnottinin 4'-O-β-ᴅ-glucopyranoside (**4**), 7-methoxy isoarnottinin 4'-O-rutinoside (**5**), oxypeucedanin hydrate-3'-O-β-ᴅ-glucopyranoside (**6**), 1-methylethyl 6-O-D-apio-β-ᴅ-furanosyl-β-ᴅ-glucopyranoside (**7**), and cnidioside A (**8**) were evaluated for antityrosinase and anticholinesterase activities for the first time in this study. The chloroform extract demonstrated selective inhibitory activity against BChE. It could be a natural source for helping the development of novel BChE inhibitor drugs with further research for the treatment of AD. Coumarins and furanocoumarins draw attention as active principles for the activity results of the extracts.

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Original Article

Effect of *Melaleuca alternifolia* oil on cytotoxicity and neuropeptide y gene expression

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ABSTRACT

Background and Aims: This study aims to investigate the effect of tea tree oil (TTO), which is an oil produced by steam distillation of the *Melaleuca alternifolia* (Maiden & Betche) Cheel plant terminal branches and leaves, on changes in neuropeptide Y (NPY) gene expression in SH-SY5Y neuroblastoma cells.
Methods: The first stage of this study investigates the cytotoxic/proliferative effects of TTO solutions prepared at different concentrations on the SH-SY5Y cell line using the tetrazolium reduction (MTT) assay. The next stage analyzes the effects of the determined TTO concentrations on NPY gene expression using the real-time polymerase chain reaction (qPCR) method.
Results: TTO concentrations prepared at a ratio of 1:32, 1:64, and 1:28 (v/v) showed statistically significant effects on cells in the cytotoxicity test and were used in the gene expression analysis. The highest significant gene expression change was seen in the cells in which the TTO solution had been applied at a ratio of 1:64 for 24 hours. NPY gene expression in these cells was determined to have increased 2.24 times compared to control cells.
Conclusion: Upon evaluating the results from the MTT assay and gene expression analysis, the solutions of the cells prepared at different TTO ratios were determined to have caused changes in gene expression. Future studies will be able to reveal all affected molecular pathways by increasing research involving TTO.

Keywords: Gene expression, Neuroblastoma, NPY, SH-SY5Y, Tea tree oil

INTRODUCTION

Tea tree oil (TTO) is an essential oil obtained mainly from the Australian endemic plant *Melaleuca alternifolia* (Maiden & Betche) Cheel. TTO is produced through steam distillation of the terminal branches and leaves of *M. alternifolia*. Used largely for its anti-microbial properties, TTO has also been included as a major ingredient in many formulations used to treat skin infections. It is widely available in the Australian, European, and North American markets and is marketed as a medicine for a variety of ailments (Carson, Hammer, & Riley, 2006). TTO consists of terpene hydrocarbons to which monoterpenes, sesquiterpenes, and alcohols are attached. TTO has a relative density of 0.885-0.906 g/mL at 25ºC (Kumari, 2013), is only slightly soluble in water, and is miscible with non-polar solvents (Carson, et al., 2006). Studies conducted between 1940-1980 in the literature have revealed TTO's anti-bacterial (Atkinson & Brice, 1955; Low, Rawal & Griffin, 1974) and later its anti-fungal (Inouye et al., 1998; Inouye et al., 2000; Inouye, Uchida, & Yamaguchi, 2001) effects. Although studies are found on the anti-microbial and anti-inflammatory properties of TTO, few studies have been done regarding the oil's toxicity and safety. Some cases of toxic effects have been reported as a result of the dermal application of TTO on cats and dogs. Typical observed symptoms are muscle tremors, incoordination, weakness, and depression (Villar, Knight, Hansen, & Buck,1994). Another

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study that dermally applied TTO on dogs and cats showed 443 cases to cause depression, weakness, coordination disorder, and muscle tremors (Khan, McLean, & Slater, 2013). One case study showed a 4-year-old child who'd swallowed a small amount of TTO to have come down with ataxia and loss of consciousness (Morris, Donoghue, Markowitz, & Osterhoudt, 2003).

Neuropeptide Y (NPY) is a peptide found in the autonomic nervous system and brain and is associated with depression, anxiety, obesity, learning and memory, epilepsy, sleep, and circadian rhythms. NPY has recently received much attention as an endogenous anti-epileptic and anti-depressant agent, because drugs with anti-epileptic or mood-stabilizing properties can reduce or increase the seizure threshold by changing NPY concentrations. NPY is also a tumor biomarker for neuroblastomas (Farrelly, Savage, O'Callaghan, Toulouse, & Yilmazer-Hanke,2013). This study has chosen to use the SH-SY5Y (ATCC® CRL-2266™) cell line that originates from malignant neuroblastoma and expresses epithelial morphology because it contains many neuron cells responsible for NPY synthesis (Kitlinska, 2007; Silva, Cavadas, & Grouzmann, 2002).

As seen, a few studies have examined TTO in the literature, and it has become an increasingly popular topical antiseptic found in many products in recent years. Due to its anti-fungal and anti-microbial properties, TTO is applied in the cosmetics industry without a warning label on market products used for various purposes, especially acne treatment and fungal infections, and people use it without being aware of it. However, upon examining the limited number of studies in the literature, TTO is seen to have a potential toxicity. In addition to its toxicity, case studies are also available in the literature that have shown this component to cause acute ataxia in humans, as well as scientific articles that have shown it to act as a depression-triggering agent when used long-term.

Investigating the potential toxicity of TTO, which has a wide range of uses in the cosmetics industry from skin creams to perfumes and soaps, and showing whether it affects the expression of genes involved in various cellular processes are important for public health.

This study investigates the effects of TTO, which is used in various cosmetic and ointment formulations due to its properties, with regard to neuroblastoma cells using cytotoxicity studies and NPY gene expression analyses. The study aims to contribute to the literature in this way by revealing the cytotoxicity of TTO and its effect on NPY gene expression, about which insufficient information is found.

MATERIALS AND METHODS

Mammalian cell culture and cytotoxicity assay

The cytotoxic activity of different concentrations of TTO (purchased from Sigma-Aldrich [Cat. No. W390208]) was assessed on the human neuroblastoma cell line (SH-SY5Y, ATCC CRL-2266). The SH-SY5Y cell line was obtained from Associate Professor Belkıs Atasever Arslan (Üsküdar University, Faculty of Engineering and Natural Sciences) as a gift.

The study uses the mammalian cell culture method alongside the tetrazolium reduction (MTT; Sigma, M-5655) assay, as previously described by Kaya, Atasever-Arslan, Kalkan, Gür, & Ülküseven,. (2016). The cell culture was incubated 24 h before each treatment. 10 µL of sterile 0.5% v/v DMSO-DMEM (Gibco, 41966) was used in place of TTO as a negative control, and the cell viability for this sample was regarded as 100%. Cells that were treated with different concentrations of TTO were incubated for 24 h and 48 h. The MTT assay results are given in the Figure 1.

The cell viability was calculated as the percentage of viable cells in the experimental group (exp.) versus the untreated (negative) control group (cont.) using the following formula, where A = absorbance of related groups:

$$\text{Cell viability (\%)} = (A_{exp.} / A_{cont.}) \times 100 \quad (1)$$

Statistical analysis of cytotoxicity assay

Two independent experiments with at least three repeats were carried out, and the results were evaluated using the program GraphPad Prism® 8. One-way analysis of variance (ANOVA) and Dunnett's test were used in order to determine the differences between the groups. The limit of significance was accepted as $p < 0.5$.

Total RNA isolation and gene expression analysis with Real-Time PCR

A commercial Biobasic E210 RNA Mini Preps Kit (Markham ON, Canada) was used for total RNA isolation, with the kit's recommended procedures being followed. The obtained RNAs were stored at -80°C. Purity and concentration measurements for all the RNAs obtained from the SH-SY5Y cells were performed with the Implen NanoPhotometer (CA, USA) instrument.

The OneScript cDNA Synthesis Kit (BC, Canada) was used for cDNA synthesis based on the isolated total RNA. Blastaq 2x qPCR MasterMix (BC, Canada) was used for the real-time polymerase chain reaction (qPCR) method. This kit's recommended procedures were followed.

The GAPDH housekeeping gene was used as a control gene. The primer sequences of the target NPY gene and GAPDH control gene are given in Table 1.

Table 1. Primer sequences of NPY and GAPDH genes.

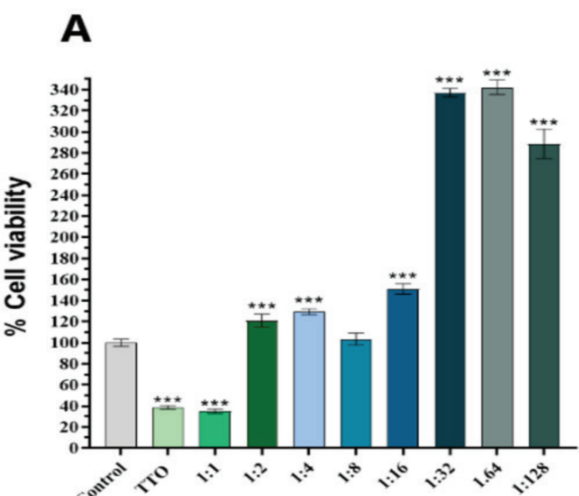
Gene	Forward Primer	Reverse Primer	Product Size
NPY	3'- GAGTTTGGGCAAGAAGGGAGA -5'	3'- GCTCCACCTGAAAACTTCGC -5'	156 bp
GAPDH	3'- AGGGCTGCTTTTAACTCTGGT -5'	3'- CCCCACTTGATTTTGGAGGGA -5'	425 bp

RESULTS

Cell viability effects of *M. alternifolia* oil and its serial dilutions on SH-SY5Y Cells

This study has investigated the effects of TTO and its serial dilutions (different concentrations) on the cell viability of human neuroblastoma cell lines (SH-SY5Y).

The results obtained as a result of the 24 h exposure of SH-SY5Y cells with TTO prepared in pure and other various dilution ratios are given in Figure 1A. The highest test concentration (Absolute TTO) of TTO was applied for 24 h, and its inhibition percentage was 61.23 ± 12.53%. The cytotoxic effect of the 1:1 dilution was calculated as 64.95 ± 12.52%. The proliferative effects of the six different dilutions [1:2, 1:4, 1:16, 1:32, 1:64, and 1:128(v/v)] were determined respectively as 121 ± 12.53%, 129 ± 12.52%, 150.96 ± 12.44%, 337.3 ± 14%, 342± 12.5%, and 288 ± 14.02%. Neither cytotoxic nor proliferative activity was observed in the other dilution (1:8; $p > 0.05$).



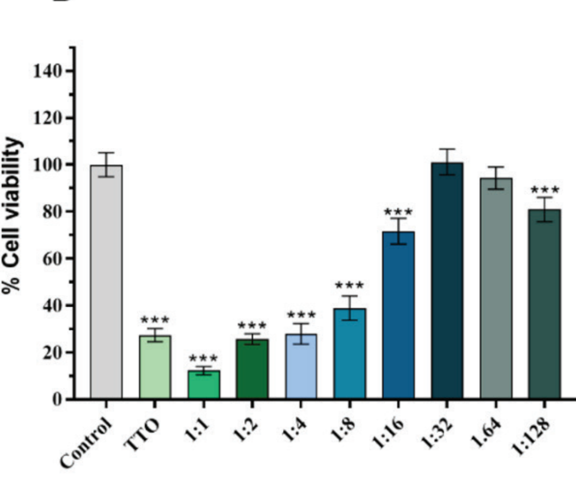


Figure 1. Comparing the effects of TTO applied on SH-SY5Y cells at different rates by volume for 24 h (A) and 48 h (B) regarding % viability (***p < 0.001, vertical bars show standard deviation values).

The results obtained after 48 h of exposure to SH-SY5Y cells with pure and prepared TTO at different dilution ratios are given in Figure 1B. The highest test concentration was TTO for 48 h, and its inhibition percentage was 72.64 ± 10.54 %. The cytotoxic effect of the six dilutions [1:1, 1:2, 1:4, 1:8, 1:16, and 1:128 (v/v)] were determined respectively as 87.75 ± 9.83%, 74.30 ± 9.84, 72.05 ± 9.83, 61.18 ± 9.84, 28.31 ± 9.83, and 19.07 ± 9.87%. Neither cytotoxic nor proliferative activity was observed in the other dilutions [1:32 and 1:64 (v/v); $p > 0.05$].

The TTO concentrations of 1:32, 1:64, 1:128 (v/v) as determined according to the cytotoxicity assay were given to the cells for 24 h and 48 h. After incubation with TTO, the total RNA isolation from the cells was performed.

Gene expression analysis

NPY gene expression levels were determined in cells exposed to different TTO concentrations for different durations. The rates of gene expression change (fold change values) determined after the $2^{-\Delta\Delta Ct}$ analysis are given in Table 2.

Table 2. Fold change values of target gene according to different TTO concentrations and durations.

Sample	Fold Change
TTO 1:32 24 h	1.14
TTO 1:32 48 h	0.45
TTO 1:64 24 h	2.24
TTO 1:64 48 h	0.40
TTO 1:128 24 h	0.13
TTO 1:128 48 h	0.01

The fold change values of NPY gene expression in cells exposed to TTO at a ratio (v/v) of 1:32 for 24 h and 48 h, were 1.14 and 0.45, respectively. The fold change values of NPY gene expression in cells exposed to TTO at a ratio of 1:64 for 24 h and 48 h, were 2.24 and 0.40, respectively. The fold change values of NPY gene expression in cells exposed to TTO at a ratio of 1: 128 for 24 h and 48 h, were 0.13 and 0.01, respectively.

According to the real-time PCR results, the 24 h 1:32 and 1:64 TTO applications were observed to have a greater effect on NPY gene expression compared to the other concentrations.

DISCUSSION

NPY regulates the behavioral consequences of stress through its activities in the brain (Heilig, 2004). One of the reasons why the behavioral anti-stress effects of NPY are important is that similar effects have been observed in a wide variety of animal models (Fendt & Fanselow, 1999; Sajdyk, Vandergriff, & Gehlert,1999). This indicates the potential effect of NPY on behavioral stress responses to be a common mechanism in many organisms.

Behavioral studies in genetically modified animals also support this hypothesis, with studies observing increased emotionality upon inactivation of NPY transmission, whereas the opposite

was observed when NPY signaling was over-activated (Heilig, 2004). Studies on rats (Stogner & Holmes, 2000) and mice (Redrobe, Dumont, Fournier, & Quirion, 2002) have shown the central NPY signals to have an anti-depressant-like effect.

With regard to the suggested organization of the corticotropin releasing factor (CRF) and NPY signaling process within the amygdala concerning fear and stress responses, various stress factors are seen to initiate a rapid release of CRF in the amygdala and facilitate the emergence of the stress response. NPY release, which begins at a later stage, is thought to mediate the termination of the acute response or act as a coping mechanism during repeated/prolonged exposure to stress (Heilig, 2004).

In the brain, some regions in the septum, especially the amygdala, hippocampus, and locus coeruleus are involved in regulating the anti-stress effects of NPY (Heilig & Murison, 1987; Naveilhan et al., 2001).

Studies are also found to have shown NPY to be involved in the neuroinflammation process (Álvaro et al., 2007; Barnea, Roberts, Keller, & Word, 2001). NPY and neuropeptide Y (Y1) receptor levels are elevated following microglia activation, as demonstrated in an endotoxin-mediated model of inflammation in a microglial cell line (Ferreira et al., 2010). This increase in NPY gene expression is thought to be a feedback mechanism for preventing the inflammatory response (Ferreira et al., 2010). *In vitro* studies have shown NPY to inhibit microglial motility through Y1 receptor activation, to inhibit phagocytosis, and to limit the effect of the inflammatory response by reducing proinflammatory cytokines and reactive oxygen species production (Duarte-Neves, Pereira de Almeida, & Cavadas, 2016).

These data in the literature reveal the potential the NPY system has to be a target for pharmacological treatments of stress-related disorders, including anxiety and depression.

Although studies on TTO in this area are limited in the literature, an examination of current studies shows TTO application to reduce tumor sizes by 80% in mice with glioblastoma (Arcella & Sanchez, 2021). In line with the results obtained from cell viability tests, the 24 h and 48 h exposures were observed to cause serious differences in cell viability when compared to similar rates, with the 24 h application of the TTO mixture prepared by diluting at a ratio of 1:2 showing a 21% proliferative effect, while the 48 h application showed a 74.30% cytotoxic effect. A similar situation was observed in the 24 h (29% proliferative) and 48 h (72.05% cytotoxic) applications prepared with the 1:4 dilutions. While the 24 h application of the TTO mixture prepared at a ratio of 1:128 (v/v) had a proliferative effect of 88% on the SH-SY5Y cells, the 48 h application showed a low toxic effect (19.07%). These results emphasize the importance of exposure time regarding the cytotoxic or proliferative effect of the relevant chemical.

In addition, when examining the results obtained from the 24 h exposure period, the inductive or inhibitory effect of the solutions obtained by diluting the same substance at different percentages with regard to cell proliferation did not vary linearly based on the dilution rate. This situation is explained by the phenomenon referred to in the literature as the hormetic dose response, which is expressed as different effects in living things as a result of different chemicals being taken into the cell/living body at varying amounts. Within the scope of this study, different proliferative or cytotoxic effects were determined for different application rates of the same substance. The literature has also shown regarding different cell culture studies that different application concentrations of the same molecule can cause different responses in a cell (Bao, Wang, Zhou, & Sun, 2014; Hayes, 2007; Wang, Calabrese, Lian, Lin, & Calabrese, 2018).

When evaluating the real-time PCR results, the 24 h 1:32 and 1:64 TTO applications were observed to have a greater effect on NPY gene expression compared to the other concentrations. While TTO at a ratio of 1:32 and applied for 24 h increased NPY gene expression 114%, TTO at a ratio of 1:64 increased the NPY gene expression 224% in the experiment group, both compared to the control group.

The data obtained within the scope of this study is thought to contribute to the literature on TTO, which has wide daily use through various drugs and cosmetic products. The study has determined the inducing and inhibitory effects of TTO and solutions prepared by diluting it with various ratios of SH-SY5Y cells. The study also investigated the effect of selected concentrations on NPY gene expression. When considering the cellular mechanisms in which the NPY gene takes an active role, investigating the effect of TTO on NPY gene expression is considered important. According to the results of the cytotoxicity test, TTO was additionally determined to inhibit cell proliferation at various concentrations.

CONCLUSION

The motivation underlying this study was to determine the effects of TTO on cytotoxicity and NPY gene expression. Excessive use of this substance in humans, consciously or unconsciously, is thought to be able to result in toxicity. As the present study shows, the use of TTO at certain ratios changes gene expression and affects molecular processes. Increasing the number of studies on TTO, which has a limited number of publications in the literature, determining the cytotoxicity of TTO with regard to different cells, and investigating its effect on gene expression will shed light on future studies.

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Original Article

An investigation and comparison of concentration change in simulated body fluid medium conditions of the Calcium element in 27 different *Salvia* species

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ABSTRACT

Background and Aims: In this study, calcium (Ca) element concentration changes of *Salvia* species in simulated body fluid (SBF) medium conditions were investigated and the results in these medium conditions were compared with each other.
Methods: *Salvia* species samples were air-dried and ground into powder. *Salvia* species was prepared as a herbal tea. Prepared teas were left in three different SBF medium conditions. The samples were analyzed using Flame Atomic Absorption Spectroscopy (FAAS) method to determine the Ca absorptions. Also, a correlation analysis of the results obtained in three different SBF medium conditions and the species in simulated gastric fluid (SGF) medium conditions was performed.
Results: When the SBF medium conditions were compared, it was determined that the highest Ca absorption of all *Salvia* species occurred in the SGF conditions. Thus, it can be said that the Ca in plants and foods occurs in the gastric fluid medium and its absorption occurs there. When the result of the correlation analysis was evaluated, it was determined that there was a stronger correlation between the SGF and simulated intestinal fluid (SIF) mediums compared to other mediums.
Conclusion: The Ca absorption was determined according to what remained in the SGF the most. Based on the results obtained from SBF medium conditions, it can be said which element is taken in which body fluid medium. Information on the differences between samples belonging to different SBF medium conditions was not obtained. However, when the simulated fluid medium conditions were evaluated individually, information was obtained for two or more samples.

Keywords: Calcium, FAAS, *Salvia*, SBF

INTRODUCTION

The Lamiaceae family, also known as Labiatae, is represented by more than 245 genera and 7886 species worldwide (Celep & Dirmenci, 2017). Members of this family include medicinal and aromatic species of commercial importance. These species are *Salvia* L., *Satureja* L., *Origanum* L., *Thymus* L., etc. (Kaya, Başer, Satil, & Tümen, 2000; Kurkcuoglu, Turnen, & Baser, 2001; Satıl, Ünal, & Hopa, 2007). In the flora of Turkey, this family is represented by 45 genera, 558 species, and 742 taxa (Yılar, Bayar, & Onaran, 2019).

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The genus *Salvia* belongs to the *Lamiaceae* family and is represented by more than 1000 species worldwide (Al Jaber, 2016; Gedik, Kiran, Emre, & Kursat, 2016; Nickavar, Rezaee, & Nickavar, 2016; Asadi-Samani, Khaledi, Khaledi, Samarghandian, & Gholipour, 2019; Coşge Şenkal, 2019; Güzel et al., 2019; Moridi Farimani, Miran, & Ebrahimi, 2019). This genus is distributed across Central and South America (500 species), West Asia (200 species), and East Asia (100 species) (Kahraman & Doğan, 2010; Büyükkartal, Kahraman, Çölgeçen, Doğan, & Karabacak, 2011; Kahraman, Doğan, & Celep, 2011). It is represented by 101 species in the flora of Turkey and 53 of them are endemic (Firat, 2020).

The species of this genus and the essential oils obtained from them are used in herbal tea, culinary herb, food preservatives, food flavorings, cosmetics, perfumery, and the pharmaceutical industry (Senatore, Formisano, Arnold, & Piozzi, 2005; Tosun et al., 2009; Kahraman, Celep, & Doğan, 2010; Nickavar et al., 2016; Güzel et al., 2019; Kahnamoei et al., 2019). Species of this genus are used to treat various diseases in traditional medicine in various parts of the world for wound healing, stomach ailments, alleviation of abdominal pain, liver and rheumatic pains, analgesic, antirheumatic, antioxidant, antibacterial, antimicrobial, antitumor, antidementia, cytotoxic, antiviral, carminative, diuretic, hemostatic, spasmolytic, sedative, as an antiseptic, in the treatment of hepatitis, menstrual disorders, in the treatment of colds, sedatives, stimulants, tonics, in the treatment of fever, bronchitis, tuberculosis, obesity, diabetes, depression, dementia, and menstrual disorders (Altun, Ünal, Kocagöz, & Gören, 2007; Kahraman, Celep, Doğan, & Bagherpour, 2010; Öztekin, Başkan, Kepekçi, Erim, & Topçu, 2010; Al-Qudah, Al-Jaber, Abu Zarga, & Abu Orabi, 2014; Al Jaber, 2016; Hegazy et al., 2018; Bağcı, Akbaba, Maniu, Ungureanu, & Hritcu, 2019; Bakir et al., 2020). Also, in folk medicine, *Salvia* species have been used since ancient times as memory-enhancing and neuroprotective agents (Çulhaoğlu, Hatipoğlu, Dönmez, & Topçu, 2015). The *Salvia* species in Turkey are known as "Adaçayı", "Çalba", "Şalba" and "Dağ çayı" and these species are used as herbal tea in folk medicine (Erdogan-Orhan, Baki, Şenol, & Yilmaz, 2010; Bağcı et al., 2019; Utsukarci et al., 2019). In Turkish folk medicine, *Salvia* species are used as antibacterials, antiseptics, diuretics, carminatives, spasmolytics, stimulants, and in treatment of wounds, colds, and coughs (Güzel et al., 2019).

For determination of element contents and concentrations of samples in the fields of chemistry, biology, medicine, pharmacy, food, environment, and agriculture various methods are used such as Atomic Absorption Spectrometry (AAS), FAAS, Graphite Furnace Atomic Absorption Spectrometry (GF-AAS), Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES), Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Instrumental Neutron Activation Analysis (INAA), and X-ray Fluorescence Spectrometry (XRF) (Pytlakowska, Kita, Janoska, Połowniak, & Kozik, 2012; Szymczycha-Madeja, Welna, & Zyrnicki, 2013; Zhang et al., 2015; Targan, Yelboğa, & Cittan, 2018; Tunay et al., 2020).

Mineral elements are of unique and versatile importance to both plants and humans. Minerals are found in plants as ions, inorganic and organic salts. Mineral elements are grouped into macro, micro, and ultra-micro elements. Macro-elements are generally considered as minerals of which the body needs more than 100 mg per day. These elements are C, H, O, N, P, K, Ca, Mg, Na, and S (Umaz, 2021). Ca is responsible for bone formation and metabolism, vascular contraction and vasodilation, muscle function, nerve conduction, intracellular signaling, and hormonal secretion. It also provides strength to bones and teeth with phosphate (Ross, Taylor, Yaktine, & Del Valle, 2011; Lippert, 2020; Shkembi & Huppertz, 2022).

Ca is absorbed in the ionized form (Ca^{2+}) in the gastrointestinal tract (a long tubular structure between the mouth and anus and a system that includes many organs associated with this structure and whose main task is digestion) (Shkembi & Huppertz, 2022). Ca is absorbed by active transport (intercellular) and passive diffusion (extracellular) across the intestinal mucosa. Active transport of the Ca is dependent on the action of calcitriol and the intestinal vitamin D receptor (VDR). Passive diffusion involves the movement of calcium between mucosal cells. this diffusion is dependent on luminal: serosal electrochemical gradients (Ross et al., 2011).

This study has the distinction of being the first in terms of examining where the Ca element in the species is absorbed under body fluid medium conditions and the concentration change. In this study, 27 different *Salvia* species used for herbal tea, medicinal, and various purposes were prepared as herbal tea and left in three different SBF mediums. The concentration variation of the Ca element, which is important for plants and humans, was investigated and compared in SBF medium conditions. Correlation analysis between the species of Ca in SGF medium conditions was performed. In addition, in order to determine whether the correlation coefficients between the obtained results were the same or not, the correlation coefficients on the basis of Ca in different SBF medium conditions were calculated and compared.

MATERIALS AND METHODS

Herbal materials

Herbarium samples of 27 *Salvia* species collected from different parts of Turkey were prepared to be placed in the herbarium and dried in accordance with the purpose of the study. Dried species were identified and preserved by Mehmet Fırat to be placed in Van Yüzüncü Yıl University, Faculty of Science Herbarium (VANF) (Table 1).

Chemicals and reagents

In the study, analytical grade chemicals were used for the preparation of three different SBF medium conditions. NaCl (EMSURE, for Analysis), $MgCl_2.6H_2O$ (for Analysis), CH_3COOK (EMSURE, for Analysis), Lactic acid (EMPROVE), NaBr (EMPROVE), $CaCl_2$ (EMSURE ACS reagent), Pepsin (Biochemistry) for Analysis), KH_2PO_4 (for EMSURE Analysis), D(+)-Fructose (for Biochemistry), and NH_4OH (EMSURE for Analysis) were purchased from Merck (Germany). D(+)-Glucose (anhydrous), NaOH (ACS reactive pellet), HNO_3 (70%), H_2O_2 (34.5-36.5%), $CuCl_2.2H_2O$ (Reactive), Urea (ACS reactive), HCl (≥ 37% ACS reagent) was purchased from Sigma Aldrich (Germany). $CaCl_2.2H_2O$ (ACS reagent), H_3PO_4 (≥

Table 1. Herbarium number, collection places, and collection times of plants belonging to 27 different *Salvia* species.

Plant species	Species Codes	Gathering Places	Harvesting Times	Herbarium Number
Salvia blepharochlaena Hedge & Hub.-Mor.	**S1**	Nevşehir	2014	M. FIRAT 32102 (VANF)
Salvia brachyantha (Bordz.) Pobed. subsp. *brachyantha*	**S2**	Van	2015	M. FIRAT 32469 (VANF)
Salvia candidissima Vahl subsp. *candidissima*	**S3**	Van-Gürpınar	2015	M. FIRAT 32092 (VANF)
Salvia ceratophylla L.	**S4**	Nevşehir	2015	M. FIRAT 32174 (VANF)
Salvia cerino-pruinosa Rech.f. var. *cerino-pruinosa*	**S5**	Elazığ	2015	M. FIRAT 32539 (VANF)
Salvia cerino-pruinosa Rech.f. var. *elazigensis* Kahraman, F.Celep & Dogan	**S6**	Elazığ	2015	M. FIRAT 32539 (VANF)
Salvia divaricata Montbret & Aucher ex Benth.	**S7**	Erzincan-Kemah	2017	M. FIRAT 33829 (VANF)
Salvia hydrangea DC. ex Benth.	**S8**	Kars-Kagızman	2014	M. FIRAT 30696 (VANF)
Salvia hypargeia Fisch. & C.A.Mey.	**S9**	Van	2015	M. FIRAT 31173 (VANF)
Salvia indica L.	**S10**	Hakkari	2015	M. FIRAT 32455 (VANF)
Salvia kronenburgii Rech. f.	**S11**	Van-Gürpınar	2014	M. FIRAT 30650 (VANF)
Salvia kurdica Boiss. & Hohen. ex Benth.	**S12**	Şırnak	2014	M. FIRAT 32614 (VANF)
Salvia limbata C.A.Mey.	**S13**	Van	2014	M. FIRAT 30660 (VANF)
Salvia macrochlamys Boiss. & Kotschy	**S14**	Van	2014	M. FIRAT 30907 (VANF)
Salvia microstegia Boiss. & Balansa	**S15**	Hakkari	2015	M. FIRAT 32472 (VANF)
Salvia montbretii Benth.	**S16**	Diyarbakır	2015	M. FIRAT 32463 (VANF)
Salvia multicaulis Vahl.	**S17**	Van	2014	M. FIRAT 30656 (VANF)
Salvia pachystachys Trautv.	**S18**	Van	2015	M. FIRAT 30878 (VANF)
Salvia pinnata L.	**S19**	Diyarbakır	2014	M. FIRAT 31318 (VANF)
Salvia pseudeuphratica Rech.f.	**S20**	Elazığ	2015	M. FIRAT 32584 (VANF)
Salvia sclarea L.	**S21**	Van-Bahçesaray	2014	M. FIRAT 30921 (VANF)
Salvia siirtica Kahraman, Celep & Doğan	**S22**	Hakkari	2014	M. FIRAT 30755 (VANF)
Salvia spinosa L.	**S23**	Mardin	2016	M. FIRAT 30908 (VANF)
Salvia suffruticosa Montbret & Aucher ex Benth.	**S24**	Van	2014	M. FIRAT 30657 (VANF)
Salvia trichoclada Benth.	**S25**	Van-Çatak	2014	M. FIRAT 30658 (VANF)
Salvia viridis L.	**S26**	Adana	2015	M. FIRAT 32461 (VANF)
Salvia xanthocheila Boiss. ex Benth.	**S27**	Van	2014	M. FIRAT 30668 (VANF)

99%), NaF (98.5-100.5%) were purchased from Honeywell/Fluka (USA). Uric acid (≥99% for Biochemistry, Roth Germany), and $K_3PO_4.3H_2O$ (97%) were purchased from abcr Gmbh (Germany). The Ca standard (1000 mg/L, Plasma CAL Calibration solution SCP28AES) in FAAS measurements was used. Linearity was evaluated using the least 6-point 3-parallel matrix calibration. Calibration curves were determined with six concentrations (5, 10, 25, 50, 75, and 100 mg/L). Calibration standard solutions (5-100 mg/L) were prepared by appropriate dilution of stock Ca standard (1000 mg/L). LOD and LOQ values of the Ca element were calculated using 10 independent blank solutions.

Preparation of SBF

Preparation of simulated saliva fluid (SSF)

1.28 g NaCl was put into 1 L flask and dissolved in 500 mL ultrapure water. Then, 0.125 g $MgCl.6H_2O$, 0.095 g KCl, 1.508 g CH_3COOK, 0.167 g $CaCl_2$, 0.386 g $K_3PO_4.3H_2O$, 0.0042 g NaF, and 0.05 mL H_3PO_4 were added to the solution and mixed. The residue and turbidity formed in the solution were clarified by adding 1 drop of H_3PO_4. Then, ultrapure water was added and mixed so that the final volume of the solution was 1 L. The solution was mixed by adding lactic acid according to the desired pH. The pH of the solution was adjusted to 6.3 (Shannon, 1982).

Preparation of SGF

0.265 g $CaCl_2.2H_2O$ was put into a 1 L flask and dissolved in 500 mL of ultrapure water. Then, the solution was mixed by adding 0.153 g $MgCl.6H_2O$, 0.865 g KCl, 2.856 g NaCl, 0.0008 g NaBr, 0.0009 g NaF, 0.0003 g $CuCl_2.2H_2O$, 0.138 g D(+)-fructose, 0.350 g D(+)-glucose, 0.084 g Urea, 0.0084 g uric acid and 3.20 g pepsin. Then, ultrapure water was added and mixed so that the final volume of the solution was 1 L. The solution was mixed by adding 0.04 M HCl and 0.1 M NH_4OH according to the desired pH. The pH of the solution was adjusted to 1.54 (Stefaniak et. al., 2010).

Preparation of SIF

6.80 g KH_2PO_4 was put into a 1 L flask and dissolved in 500 mL of ultrapure water. Then, 0.90 g of NaOH was added to the solution and mixed. Then, ultrapure water was added and mixed so that the final volume of the solution was 1 L. The solution was mixed by adding 2 M HCI according to the desired pH. The pH of the solution was adjusted to 6.80 (Stippler et. al., 2004).

Preparation of *Salvia* species as herbal tea

0.5 g *Salvia* species was put into a 100 mL beaker and washed two times with ultrapure water to remove dust and residues. Then, 50 mL boiled ultrapure water was added to the beaker and was left to brew for 10 min. Then, the tea in the beaker was filtered with blue banded filter paper. The resulting filtrate was used in experimental studies.

Experimental designs in SBF medium of prepared herbal tea

50 mL SBF was placed in a 250 mL beaker and 50 mL of prepared herbal tea was added to it. The mixture was put on a mixer and mixed to rotate at 50 rpm. The stirring times designed for the simulated mediums were as follows: 30 seconds for SSF medium, 180 minutes for SGF medium, and 300 minutes for SIF medium. Then the mixture was filtered with filter paper and the samples were placed in screw-capped tubes to be read in the FAAS device. The above-mentioned procedures were performed separately for each SBF medium. Analysis was performed in three replicates for simulated saliva, gastric and intestinal fluid medium. In the study, 27 *Salvia* species were used and three different SBF mediums were studied based on the duration of food intake.

Table 2. FAAS instrument operating analytical conditions

Measured Element	Ca
Wavelength	422.7 nm
Slit Width	0.7 nm
Lamp Current	10 mA
Gas Flow Rate	2 L/min
Flame Height	7 mm
Flame Type	Air-Acetylene

FAAS analysis

The Ca analysis in SBF medium of species samples and prepared herbal tea was performed using FAAS (AA-7000, Shimadzu, Japan) (Table 2).

The linear range, calibration equation, correlation coefficient (R^2), limit of detection (LOD), and limit of measurement (LOQ) values are shown in Table 3. The sensitivity of the method was evaluated by determining the limits of detection (LOD) and limits of quantification (LOQ). The LOD and LOQ values were determined under FAAS conditions from 3S/m and 10S/m respectively. The S was the standard deviation of the blank and m was the slope of the calibration equation. The LOD and LOQ values in SSF, SGF, and SIF medium conditions were found as 1.440 and 4.751 mg/L; 1.309 and 4.320 mg/L; 2.524 and 8.328 mg/L, respectively. The correlation coefficients (R^2) in SSF, SGF, and SIF medium conditions were determined as 0.990, 0.995, and 0.998, respectively (Table 3).

Statistical analysis

The analysis of the species was replicated three times and the mean values of the data were used in the statistical analysis. The correlation of Ca elements in 27 *Salvia* species structures between three different SBF medium conditions was determined using linear correlation with SSPS 21 statistical package program (2012).

RESULTS AND DISCUSSION

Variation in SBF medium conditions of Ca concentration

Ca is known to be an important mineral element for plants and humans. For this reason, regarding the Ca element in the species which is used for medicinal and various purposes, it is important to know what ratio is absorbed especially in humans. This study was carried out to estimate the amount that can be taken up by the organism of an element found in the digestible materials (herbal tea, food, and medicines). When the Ca absorption in SBF medium conditions of *Salvia* species was examined, it was determined that the Ca absorption of the species varied between 118-373 mg/kg in SSF medium conditions. The Ca absorption of the S22 sample was determined to be higher than other species in SSF medium conditions. In addition, the Ca absorptions of almost all species were detected

Table 3. FAAS instrument operating analytical conditions.

Element	Linear Range (mg/L)	Calibration Equation	R^2	LOD (mg/L)	LOQ (mg/L)
SSF					
Ca	25-100	y= 0.0028104x - 0.19540	0.990	1.440	4.751
SGF					
Ca	5-75	y= 0.0038165x - 0.25492	0.995	1.309	4.320
SIF					
Ca	10-100	y= 0.0025610x - 0.20674	0.998	2.524	8.328

to be close to each other. As a result, it was determined that the Ca element was taken into the SSF medium in all 27 *Salvia* species (Table 4). When the Ca absorption between the species is compared under SSF medium conditions, it can be said that higher absorption in the S22 species gives more Ca^{2+} ions to the medium.

Table 4. Ca contents in simulated body fluid medium conditions of the species (n=3.

	SSF	SGF	SIF
Samples	**Ca (mg/kg)**	**Ca (mg/kg)**	**Ca (mg/kg)**
S1	230±27	18300±421	11096±844
S2	263±22	27511±109	**13119±123**
S3	308±19	33740±344	11352±164
S4	237±12	31838±810	11322±219
S5	275±4	37184±149	9398±65
S6	213±21	37723±166	11644±95
S7	195±22	31521±566	11256±633
S8	294±16	39783±151	12287±128
S9	214±46	37445±501	11494±678
S10	288±19	42316±184	12290±431
S11	267±19	27030±787	9307±304
S12	271±6	36991±113	9521±272
S13	296±20	40034±120	10182±438
S14	147±13	39442±513	10012±650
S15	283±18	38128±470	10302±755
S16	211±19	**62374±159**	10023±465
S17	187±14	55063±241	8415±112
S18	251±24	38177±150	8715±169
S19	330±17	38881±411	9104±120
S20	219±17	37626±405	8904±207
S21	263±21	37981±817	8976±490
S22	**373±10**	37746±766	8521±223
S23	292±19	36990±247	8521±406
S24	118±7	37668±626	9307±172
S25	210±14	37210±672	9458±209
S26	240±6	37131±158	12628±272
S27	262±6	38923±268	9120±82

The Ca absorption in the species was determined that varied between 18300-62374 mg/kg in SGF medium conditions. The Ca absorption of the S16 sample was determined to be higher than the other species in SGF medium conditions. It was determined that in all *Salvia* species, the Ca was taken into the SGF medium (Table 4).

The Ca absorption of the species was determined as varying between 8415-13119 mg/kg in SIF medium conditions. The Ca absorption of the S2 sample was determined to be higher than the other species in SIF medium conditions. It was determined that the Ca was taken into SIF medium in all *Salvia* species (Table 4).

Schwedt, Tawali, & Koch, (1998) the total content of zinc in the extraction with simulated gastric and intestinal fluid of foodstuffs has beendetermined. In simulated gastric juice, the total zinc content in Buckwheat flour, Rye flour, Potato, Pea, and Beef products was reported as 3.628, 1.222, 0.362, 0.751, and 4.654 mg/kg, respectively. In simulated intestinal juice, the total zinc content in the products was determined as 1.023, 0.130, 0.256, 0.371, and 3.562 mg/kg, respectively. It has been stated that the zinc element is mostly absorbed from the gastric juice (Schwedt et. al., 1998).

Giacomino et. al., (2014) the metal bioaccessibility of three drugs purchased in India has been investigated by extraction with solutions simulating gastric and intestinal fluids. The concentrations of the elements extracted upon contact between gastric and intestinal juices of the samples of drug C, D and F were reported as 1740 and 2170, 7640 and 5091, 1893 and 2490 mg/kg, respectively. Generally, it has been specified that the release of most of the analytes in the gastric medium was lower than that in the intestinal one (Giacomino et. al., 2014).

Wang et. al., (2019) the uptake of Fe, Zn, Ca, and Mg in gastric and intestinal fluids of acidic heteropolysaccharide (LP) from *Lycium barbarum* L. leaves have been determined. The Ca intakes in the gastric and intestinal fluids of the crude LP (LPC) have been reported as 0.19 and 0.54 mg, respectively. It has been stated that the Ca element is mostly absorbed from the intestinal juice (Wang et. al., 2019).

When Table 4 was examined, the ranking of Ca absorption of *Salvia* species in SBF medium conditions was determined as SGF > SIF > SSF. Thus, it was determined that the Ca element was absorbed most in gastric fluid and least in saliva fluid (Table 4). It can be said that the absorption of Ca in the saliva fluid being less than in other body fluids is due to the fact that the pH of the saliva is close to neutral. It has been reported in the literature that saliva has only a negligible effect on the level of mobilization of metal contaminants, as the pH of saliva is close to neutral (Giacomino et. al., 2014). When the SGF and SIF medium conditions were compared, the Ca absorption of *Salvia* species was determined as being higher in SGF medium conditions. It can be said that the most absorption of Ca in the gastric fluid medium is due to the acid pH of the gastric juice permiting a greater dissolution of the hydroxides, oxides, other salts, and organic species of the elements contained in the plant. Although the pH of the intestinal fluid was close to neutral, the Ca absorption in the species was determined as being high. Thus, it reveals that the Ca found in food, medicine, and herbal teas is absorbed in the intestine. Consequently, the Ca element, its carbonates, oxides, sulfites, and salts in plants and foods are mostly absorbed in gastric and intestinal fluid. It can be said that in human and animal bodies, Ca passes through the gastric and intestinal medium. When the Ca is evaluated in general, it can be said that it is absorbed in the gastric medium and is taken into the living body. The findings of this study showed that the Ca element in *Salvia* species was absorbed from the SGF and SIF fluid medium and was similar to the results in the literature.

Correlation analysis of SGF

It was aimed to determine whether there were differences between species according to SGF of the correlation coefficient and direction with correlation analysis. Correlation analysis for species in SGF medium conditions was used to provide information in determining whether there is a relationship in terms of Ca absorption between species that are related to each other and the relationship between species in the same region or close locations. Therefore, correlation analyses were performed for species in SGF medium conditions (Table 5 and 6). According to the results of the bivariate analysis of the samples belonging to SGF, the Pearson correlation coefficient (r) was at $p < 0.01$ confidence level; it was determined that there was a strong negative relationship in terms of Ca absorption between S5 with S4, S16 with S7, and S19 with S8 ($r = -0.999$). In addition, it was determined that there was a strong positive relationship ($r = 0.999$) in terms of Ca absorption between S26 with S21 species in the same medium conditions. When the Pearson correlation coefficients of the samples belonging to these simulated medium conditions were compared, it was determined that the strongest correlation at $p < 0.01$ confidence level was between S5 with S4, S16 with S7, S19 with S8, and S26 with S21 (Table 5).

When the $p < 0.05$ confidence level of the Pearson correlation coefficient (r) of the samples belonging to SGF was examined, it was determined that there was a strong negative relationship in terms of Ca absorption between S12 with S2, S12 with S9, S15 with S11, S17 with S12, S17 with S16, S23 with S20 and S24 with S3 ($r = -0.999$). It was determined that there was a strong negative relationship in terms of Ca absorption between S23 with S1 ($r = -0.998$), S26 with S9, and S19 with S11 ($r = -0.997$). In addition, it was determined that there was a strong positive relationship in terms of Ca absorption between S9 with S2, S17 with S7, S25 with S6 ($r = 0.999$), S17 with S2, S27 with S6, S11 with S8, S15 with S13 ($r = 0.998$) (Table 5).

Davis has been examined the close kinship of *Salvia* species with each other. He assigned a number to each *Salvia* species. According to these numbers, he said that the species between 1-7, 8-14, 15-31, 32-41, 42-47, 48-83, and 84-86 showed close kinship relationships (Table 6) (Davis, 1982). The relatedness of *Salvia* species to each other is defined by the closeness to each other of the numbers given to them. In this context, when the correlation coefficient and effect were examined, according to the results of the correlation analysis at $p < 0.01$ confidence level, a strong relationship between the species in terms of calcium absorption was determined. However, it was determined that there is no relationship between consanguinity and Ca absorption within these species (Table 6).

At the $p < 0.05$ confidence level, it was determined that there was a relationship in terms of calcium absorption between S26 (Species Kinship Code: 43) with S9 (44), S11 (38) with S8 (36), and S15 (57) with S.13 (71) which only have kinship to each other from the species which has a strong relationship with each other. As a result, it was determined that although there was a strong relationship between species in terms of calcium absorption at both $p < 0.01$ and $p < 0.05$ confidence levels, there was generally no relationship between the closely related species with each other (Table 6).

When the correlation coefficient and relationship between species collected from in the same region were evaluated, it was determined that there was only a relationship in terms of Ca absorption between S15 with S11, S24 with S3, S9 with S2, S17 with S7, S17 with S2, S27 with S6, S11 with S8, and S15 with S13 (Eastern Anatolia Region) from the species with a strong relationship with each other in the $p < 0.05$ confidence level. Dogan et. al., (2008) *Salvia* made the intra-genus classification. They divided the species into seven different sections. These sections are *Hymenosphace* Benth., *Aethiopis* Benth., *Plethiosphace* Benth., *Horminum* Benth., *Drymosphace* Benth., *Hemisphace* Benth., and *Salvia* Hedge. They grouped the species based on these sections (Table 6) (Dogan et. al., 2008). When the results of correlation analysis at $p < 0.01$ confidence level were examined at the intra-genus classification level, it was determined that there was no relationship between species in terms of Ca absorption. At the $p < 0.05$ confidence level, it was determined that there was a relationship in terms of Ca absorption only between S8 with S11 (*Hymenosphace-Hymenosphace*), S13 with S15 (*Aethiopis-Aethiopis*), and S9 with S2 (*Aethiopis-Aethiopis*) from the species with a relationship with each other (Table 6). Thus, the significant correlation between Ca element concentrations in species indicates a similar ability or presence of the same source for the *Salvia* species. The dependence between the species with the strongest correlation above can be explained by their common origin.

Correlation analysis of simulated body fluid medium conditions

It was aimed to determine whether the correlation coefficient and its direction differ in three different SBF medium conditions. For this reason, correlation analysis were performed for three different SBF medium conditions (Table 7) (Figure 1).

According to the correlation matrix of the Ca element in three different SBF medium conditions, negative correlations were detected between SGF with SIF ($r= -0.276$), SGF with SSF ($r= -0.132$)

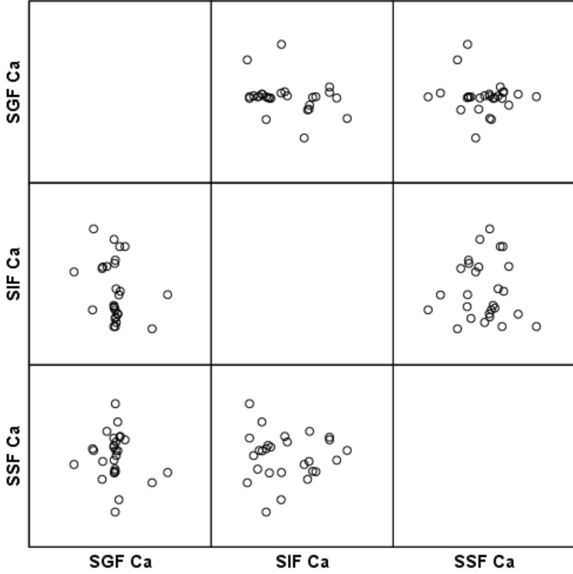

Figure 1. Scatter/Dot plot of Ca in simulated body medium conditions of the species.

Table 5. Correlation matrix for the Ca concentration of species in SGF medium conditions.

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25	S26	S27
S1	1																										
S2	0,725	1																									
S3	0,988	0,822	1																								
S4	0,977	0,561	0,932	1																							
S5	-0,978	-0,566	-0,935	**-0,999**[a]	1																						
S6	-0,925	-0,408	-0,855	-0,985	0,984	1																					
S7	0,794	0,994	0,878	0,646	-0,650	-0,503	1																				
S8	0,900	0,953	0,956	0,786	-0,790	-0,667	0,98	1																			
S9	0,702	**0,999**[b]	0,803	0,533	-0,538	-0,378	0,99	0,942	1																		
S10	-0,855	-0,262	-0,765	-0,946	0,944	0,988	-0,363	-0,543	-0,230	1																	
S11	0,926	0,932	0,973	0,823	-0,827	-0,712	0,965	**0,998**[b]	0,919	-0,595	1																
S12	-0,739	**-0,999**[b]	-0,834	-0,578	0,583	0,427	-0,996	-0,959	**-0,999**[b]	0,282	-0,939	1															
S13	-0,961	-0,886	-0,992	-0,880	0,883	0,784	-0,931	-0,985	-0,871	0,679	-0,994	0,896	1														
S14	0,254	-0,482	0,103	0,455	-0,450	-0,603	-0,386	-0,193	-0,511	-0,719	-0,131	0,463	0,022	1													
S15	-0,944	-0,912	-0,983	-0,852	0,855	0,747	-0,950	-0,993	-0,898	0,635	**-0,999**[b]	0,920	**0,998**[b]	0,079	1												
S16	-0,793	-0,994	-0,877	-0,644	0,649	0,502	**-0,999**[a]	-0,979	-0,990	0,361	-0,965	0,996	0,930	0,388	0,950	1											
S17	0,769	**0,998**[b]	0,858	0,615	-0,619	-0,468	**0,999**[b]	0,971	0,995	-0,325	0,954	**-0,999**[b]	-0,915	-0,423	-0,937	**-0,999**[b]	1										
S18	0,175	0,805	0,324	-0,040	0,034	0,213	0,737	0,586	0,824	0,362	0,534	-0,792	-0,439	-0,908	-0,490	-0,739	0,764	1									
S19	-0,895	-0,956	-0,953	-0,779	0,782	0,658	-0,982	**-0,999**[a]	-0,946	0,533	**-0,997**[b]	0,962	0,983	0,204	0,992	0,982	-0,973	-0,596	1								
S20	0,995	0,652	0,968	0,993	-0,994	-0,958	0,729	0,852	0,627	-0,902	0,883	-0,668	-0,929	0,349	-0,906	-0,728	0,701	0,076	-0,846	1							
S21	-0,638	-0,993	-0,748	-0,458	0,463	0,297	-0,975	-0,910	-0,996	0,145	-0,882	0,990	0,825	0,583	0,856	0,975	-0,983	-0,870	0,914	-0,558	1						
S22	-0,705	-0,023	-0,588	-0,840	0,837	0,922	-0,129	-0,326	0,011	0,971	-0,385	0,044	0,483	-0,865	0,431	0,127	-0,089	0,575	0,315	-0,772	-0,097	1					
S23	**-0,998**[b]	-0,679	-0,976	-0,989	0,989	0,947	-0,753	-0,870	-0,654	0,886	-0,900	0,694	0,942	-0,316	0,921	0,752	-0,726	-0,111	0,864	**-0,999**[b]	0,587	0,749	1				
S24	-0,991	-0,811	**-0,999**[b]	-0,939	0,941	0,865	-0,868	-0,950	-0,791	0,777	-0,968	0,823	0,990	-0,122	0,980	0,868	-0,848	-0,305	0,947	-0,973	0,735	0,604	0,980	1			
S25	-0,916	-0,388	-0,844	-0,981	0,979	**0,999**[b]	-0,484	-0,650	-0,357	0,991	-0,696	0,407	0,770	-0,621	0,732	0,482	-0,448	0,234	0,641	-0,952	0,275	0,930	0,940	0,854	1		
S26	-0,647	-0,994	-0,756	-0,469	0,474	0,308	-0,977	-0,915	**-0,997**[b]	0,157	-0,887	0,992	0,832	0,573	0,862	0,978	-0,985	-0,864	0,919	-0,568	**0,999**[a]	-0,080	0,597	0,743	0,287	1	
S27	-0,948	-0,468	-0,888	-0,994	0,993	**0,998**[b]	-0,559	-0,715	-0,438	0,975	-0,757	0,487	0,824	-0,549	0,790	0,558	-0,526	0,147	0,706	-0,975	0,359	0,894	0,967	0,897	0,996	0,371	1

[a] Correlation is significant at the 0.01 level, [b] Correlation is significant at the 0.05 level.

Table 6. The relatedness of *Salvia* species to each other and the in-genus classification.

Species	Species Codes	Species Kinship Code	Sections
Salvia blepharochlaena	S1	34	*Hymenosphace* Benth.,
Salvia brachyantha subsp. *brachyantha*	S2	49	*Aethiopis* Benth.,
Salvia candidissima subsp. *candidissima*	S3	66	*Aethiopis* Benth.,
Salvia ceratophylla L.	S4	53	*Aethiopis* Benth.,
Salvia cerino-pruinosa var. *cerino-pruinosa*	S5	-	*Hymenosphace* Benth.,
Salvia cerino-pruinosa var. *elazigensis*	S6	-	*Hymenosphace* Benth.,
Salvia divaricata	S7	1	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia hydrangea	S8	36	*Hymenosphace* Benth.,
Salvia hypargeia	S9	44	*Aethiopis* Benth.,
Salvia indica L.	S10	72	*Aethiopis* Benth.,
Salvia kronenburgii	S11	38	*Hymenosphace* Benth.,
Salvia kurdica	S12	6	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia limbata	S13	71	*Aethiopis* Benth.,
Salvia macrochlamys	S14	7	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia microstegia	S15	57	*Aethiopis* Benth.,
Salvia montbretii	S16	45	*Aethiopis* Benth.,
Salvia multicaulis	S17	40	*Hymenosphace* Benth.,
Salvia pachystachys	S18	27	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia pinnata L.	S19	11	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia pseudeuphratica	S20	-	*Hymenosphace* Benth.,
Salvia sclarea L.	S21	50	*Aethiopis* Benth.,
Salvia siirtica	S22	-	-
Salvia spinosa L.	S23	46	*Aethiopis* Benth.,
Salvia suffruticosa	S24	29	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia trichoclada	S25	14	*Salvia* Hedge (*Eusphace* Benth.,)
Salvia viridis L.	S26	43	*Horminum* Benth.,
Salvia xanthocheila	S27	58	*Aethiopis* Benth.,

Species kinship between 1-7, 8-14, 15-31, 32-41, 42-47, 48-83 and 84-86.

Table 7. Correlation matrix of species in simulated body fluid medium conditions.

	SGF	SIF	SSF
SGF	1		
SIF	-0.276	1	
SSF	-0.132	-0.027	1

and SSF with SIF (r= -0.027). When the correlations in SBF medium conditions were evaluated together, they were determined that SGF-SIF > SGF-SSF > SSF-SIF. When all the correlation coefficients were evaluated in general, it was determined that all SBF medium conditions were weakly correlated with each other. In addition, considering all correlation coefficients, SGF and SIF medium were detected to have stronger negative correlations than other mediums. The fact that these correlations were different from the specified SBF mediums can be explained as the result of taking other elements such as the Ca element into the medium.

CONCLUSION

Plants have medical benefits because they contain various bioactive compounds as well as mineral elements. For this reason, it is important to know the contents of mineral elements in plants and how much these elements are absorbed. In this context, this study has determined the Ca content and absorption of 27 *Salvia* species in three different SBF conditions. In addition, the correlation analysis of the obtained results has been performed.

According to the analysis results in different SBF medium conditions of the samples belonging to *Salvia* species, it has been determined that the Ca absorption in the SGF the most. Based on the results obtained from SBF medium conditions, it can be said which element is taken in which body fluid medium. In ad-

dition, it can be said that element-based synthesized drugs are a guide in the selection of the target region they want to affect.

According to the results of the bivariate analysis in SBF medium conditions, information on the differences between the samples belonging to different SBF medium conditions could not be obtained. However, when SBF medium conditions are evaluated alone, the only information that may indicate the same source or behavior is obtained for two or more items. Thus, it was determined that more complex analysis systems should be used to determine the difference in terms of the medium conditions.

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Original Article

Antioxidants prevent indomethacin-induced oxidative damage in tongue tissues of rats

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ABSTRACT

Background and Aims: Indomethacin (Indo) is an important nonsteroidal anti-inflammatory drug that has beneficial effects as a pain killer, but also has side effects. In this study, the protective effects of vitamins, selenium and ranitidine (Ran) against the side effects of Indo on tongue tissue were investigated.
Methods: Rats were divided into six groups: the control group as group I; the group given a vitamin-selenium (Vitamin C, 100 mg/kg; Vitamin E, 100 mg/kg, beta carotene, 15 mg/kg, selenium, 0.2 mg/kg) combination as group II; the group treated with Indo as group III; the group administered vitamins-selenium and Indo (25 mg/kg, single dose) as group IV; the group given Ran (150 mg/kg) as group V; and the group given Ran and Indo as group VI. The experiments were done for 3 days. Animals received vitamins-selenium and Ran for 3 days, 2 h before the Indo administration (in same dose and time). Tongue tissues were taken and homogenized.
Results: In the supernatants, glutathione levels, superoxide dismutase, catalase and glutathione-dependent enzyme activities were reduced. Protein carbonyl, lipid peroxidation levels, lactate dehydrogenase and myeloperoxidase activities were increased in the group that was given Indo. Administration of vitamins-selenium and Ran reversed the levels and activities of the tested parameter in the Indo group.
Conclusion: We suggest that vitamins, selenium and Ran have powerful protective effects on Indo-induced tongue injury in rats.

Keywords: Beta carotene, Vitamin C, Vitamin E, Indomethacin, Tongue tissue, Selenium

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) are one of the most preferred drug classes as a result of their multiple drug actions, which include analgesic, anti-inflammatory and antipyretic effects. They generally inhibit cyclo-oxygenase (COX) enzymes which convert arachidonic acid to eicosanoids like thromboxane and prostaglandins (Bacchi, Palumbo, Sponta, & Coppolino, 2012; Baker & Perazella, 2020), whose elevated expressions are associated with increased cardiovascular diseases (Radi & Khan, 2019).

Indomethacin (Indo) is one of the most commonly used NSAIDs due to its unique pain killer effect, and is an indole-acetic acid derivative (Bindu, Mazumder, & Bandyopadhyay, 2020). Despite their effectiveness and useful aspects, both Indo and other NSAIDs have been reported to cause serious gastrointestinal system and kidney injury (Varghese, Faith, & Jacob, 2009; Turkyilmaz, Coskun, Bolkent, & Yanardag, 2019a, Eraslan, Tanyeli, Güler, Kurt, & Yetim, 2020). Indo has been reported to increase free radical production by different mechanisms (Turkyilmaz, Arda Pirincci, Bolkent, & Yanardag, 2019b). The drug results in radical production either via tumour necrosis factor alpha (TNF-a) stimulation in gastric ulcer and cancer risk (Moustafa, Khoder, El-Awady,

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& Zaitone, 2013), or by facilitating the breakdown of Indo on mitochondrial functioning and oxygen uptake (Basivireddy, Jacob, & Balasubramanian, 2005). This situation has challenged scientists to come up with solutions for preventing the deteriorative effect of Indo.

Vitamin C (Vit C) and vitamin E (Vit E) are the two most important vitamins that work synergistically. Their protective effects either alone or in combination can be termed a second line of defence. They support the endogenous antioxidant defence system through the regeneration of important molecules like reduced glutathione (GSH), preventing lipid peroxidation of membrane structure, facilitating the synthesis of different hormones and neurotransmitters, improving immune systems, etc. (Wagner, Buettner, & Burns, 1996; Shaik-Dasthagirisaheb et al., 2013; Michalczyk, Czuba, Zydek, Zając, & Langfort, 2016). In addition, beta carotene, a precursor of vitamin A (Vit A), also has vital roles such as free radical neutralization, prevention of peroxidative process progression and regulation of inflammatory systems (Dragnev, Rigas, & Dmitrovsky, 2000; Valko et al., 2007; Rocha et al., 2018). Vit A has been reported to stabilize thiol groups of membranes (Rutkowski & Grzegorczyk, 2012). Selenium (Se) is an essential micronutrient whose necessity carries great importance for regulation of inflammation (Hariharan & Dharmaraj, 2020), its existence in selenoproteins like glutathione peroxidase (Cai, Zhang, & Li, 2019), and participation in redox signalling and homeostasis (Guillin, Vindry, Ohlmann, & Chavatte, 2019). Ranitidine (Ran), a histamine H_2-receptor antagonist, is effective in reducing excess gastric acid production and secretion (Ryan, Barker, & Hawcutt, 2020). Its protective effect on Indo-induced small intestinal injury was proven by Turkyilmaz et al., (2019b).

The tongue can be negatively affected for many reasons, including age, alcohol consumption, cigarette use and NSAID utilization (Baek et al., 2017; Kalogirou & Tosios, 2019; Bayrak, Arda-Pirincci, Bolkent, & Yanardag, 2021). For this reason, we directed our attention to preventing tongue damage induced by Indo via the proven protective effects of antioxidants and Ran.

MATERIALS AND METHODS

Animals

The procedure of this study was carried out according to the Local Ethics Committee on Animal Research at Istanbul University (Approval No: 2008/21). Male Sprague Dawley rats, aged 2.5-3.0 months, were chosen. All the animals had access to standard pellet chow and tap water.

Experimental design

The current study was intended to investigate the protective effects of an antioxidant combination and Ran against Indo-induced tongue injury in rats. Rats were randomly split into six groups of eight animals per group. Group I was control animals. Group II was control animals that received Vit C (100 mg/kg per day), Vit E (100 mg/kg per day), beta carotene (15 mg/kg per day) and sodium selenate (Se) (0.2 mg/kg per day) for 3 days by gavage technique. Group III was animals that received Indo at 25 mg/kg at a single dose by gavage technique (Koc, Imik, & Odabasoglu, 2008). Group IV was animals given Vit C, Vit E, beta carotene and Se over 3 days, with the final dose of this combination given 2 h before Indo administration (in the same dose and time). Group V was animals given Ran at a dose of 150 mg/kg per day for 3 days by gavage technique. Group VI was animals who received Ran in the same dose and time, and its final dose was applied 2h before Indo administration. After 6h of Indo administration, all animals were sacrificed under anaesthesia. Vit C, Se and Ran were dissolved in distilled water. Vit E was (in its acetate form) dissolved in distilled water just like Vit C. Beta carotene was prepared in sunflower oil. Indo was prepared by dissolving Tween-80 and 0.9% NaCl (1mL:9mL, respectively).

Biochemical experiments

After all animals were sacrificed, tongue tissues were taken and kept in physiological saline at -80 °C. Tongue tissues were homogenized in cold physiological saline to make up 10% homogenates (weight/volume). The homogenates were centrifuged at 20000 x g for 10 minutes. After the centrifugation procedure, collected supernatants were used to evaluate reduced glutathione (GSH; Beutler, 1975), lipid peroxidation (LPO; Ledwozyw, Michalak, Stepień, & Kadziołka, 1986) and protein carbonyl (PC; Levine et al., 1990) levels. For determining enzyme activities, the following parameters were chosen, respectively: catalase (CAT; Aebi, 1984), superoxide dismutase (SOD; Mylroie, Collins, Umbles, & Kyle, 1986), glutathione-S-transferase (GST; Habig & Jakoby, 1981), glutathione peroxidase (GPx; Wendel, 1981), lactate dehydrogenase (LDH; Bais & Philcox, 1994) and myeloperoxidase (MPO; Wei & Frenkel, 1991). The protein levels of tongue tissue homogenates were evaluated by referencing the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

Statistical analyses

Values are expressed as the "*mean ± standard deviation* (SD)" and analysed statistically using GraphPad Prism (version 6.0, GraphPad Software, San Diego, California, USA). An unpaired t-test, analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed to determine the significance of difference at $P < 0.05$.

RESULTS

The tongue GSH, LPO and PC levels of all groups are shown in Figure 1. Administration of antioxidants caused a significant decrease in GSH levels ($P < 0.01$) and insignificant elevations in LPO and PC levels of the control group. Indo reduced GSH levels but increased LPO and PC levels significantly compared to the control group ($P < 0.001$, $P < 0.01$, respectively). Antioxidants reversed these abnormal levels in the Indo group in a significant manner ($P < 0.001$, $P < 0.05$, respectively). It was determined that Ran decreased GSH and LPO levels insignificantly while its administration significantly increased PC levels as compared to the control group ($P < 0.05$). Treatment with Ran in the Indo group reversed GSH levels significantly ($P < 0.001$), while its effect on LPO and PC levels was insignificant (Figure 1).

In Figure 2, CAT, SOD, GST and GPx activities of all groups are given. According to the results, antioxidant administration increased CAT activity and decreased SOD activity insignificantly, while no differentiation was detected in GST activity when compared to the control group. In addition, a significant diminishment was deter-

mined at GPx activity of the antioxidant group as compared to the control group (*P* < 0.0001). It can be said that Indo decreased SOD, GST and GPx activities significantly compared to the control group (*P* < 0.01, *P* < 0.05, *P* < 0.0001, respectively). However, CAT activities were insignificantly affected in the Indo group as compared to the control group. The administration of antioxidants to the Indo group increased CAT, SOD and GST activities significantly (*P* < 0.01, *P* < 0.001, respectively), while GPx activities were insignificant. Ran caused a remarkable decrease in GPx activity (*P* < 0.0001), although the changesets of CAT, SOD and GST activities were insignificant when compared to the control group. Ran ameliorated all enzyme activities significantly in the Indo group (*P* < 0.001, *P* < 0.05, respectively) (Figure 2).

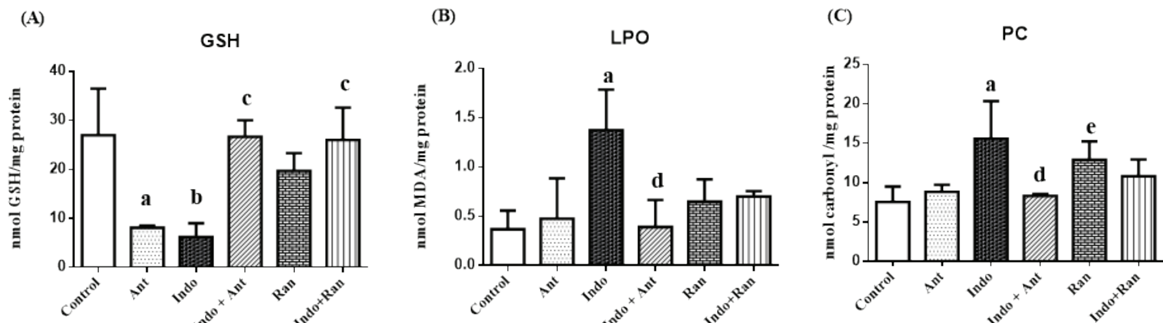


Figure 1. The tongue tissue GSH (A), LPO (B) and PC (C) levels of control and experimental groups. Values are expressed as mean ± SD. [a]*P*<0.01 vs control group, [b]*P*<0.001 vs control group, [c]*P*<0.001 vs Indo group, [d]*P*<0.05 vs Indo group, [e]*P*<0.05 vs control group. Ant: antioxidants (vitamins-selenium); GSH: reduced glutathione; Indo: indomethacin; LPO: lipid peroxidation; PC: protein carbonyl; Ran: ranitidine; SD: standard deviation.

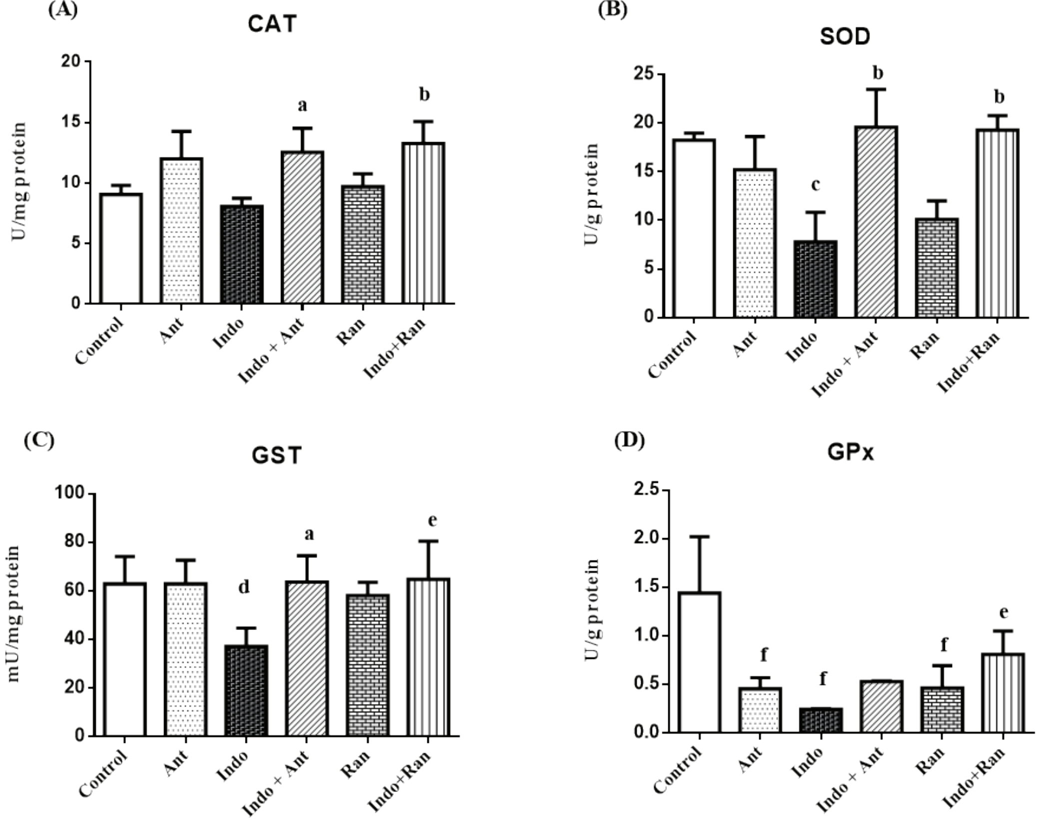
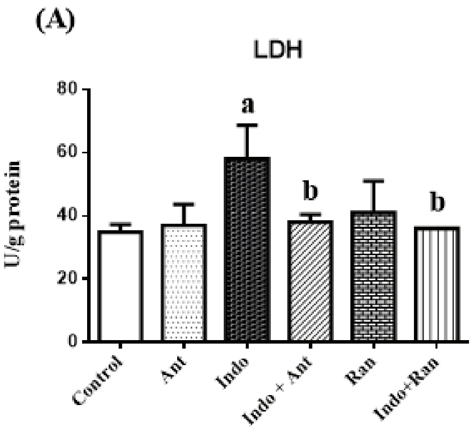


Figure 2. The tongue tissue CAT (A), SOD (B), GST (C) and GPx (D) activities of control and experimental groups. Values are expressed as mean ± SD. [a]P<0.01 vs Indo group, [b]P<0.001 vs Indo group, [c]P<0.01 vs control group, [d]P<0.05 vs control group, [e]P<0.05 vs Indo group, [f]P<0.0001 vs control group. Ant: antioxidants (vitamins-selenium); CAT: catalase; GPx: glutathione peroxidase; GST: glutathione-S-transferase; Indo: indomethacin; Ran: ranitidine; SOD: superoxide dismutase; SD: standard deviation.

The LDH and MPO activities of control and experimental rats are depicted in Figure 3. Antioxidant administration increased LDH activity and decreased MPO activity insignificantly as compared to the control group. Indo administration caused significant increases of both enzyme activities when compared to the control group ($P < 0.001$, $P < 0.0001$, respectively). Ran caused an insignificant increase in LDH activity and didn't change MPO activities of the control group. The antioxidants and Ran significantly decreased LDH and MPO activities in the Indo group ($P < 0.05$; $P < 0.0001$, respectively) (Figure 3).

caspase 3 and 9 expressions, was observed. This diminishment results in the inhibition of COX-2, a related enzyme in apoptosis and apoptosis-related caspase 3 and 9 levels. When all the possible mechanisms are taken into consideration, the elevation of reactive oxygen species (ROS) levels is inevitable. The goal of the present study was to scavenge the deleterious effect of Indo on tongue tissue like gastric and intestinal tissues.

Despite low concentrations of GSH in the biological system, it serves as one of the most effective antioxidants in many organs and tissues. It also serves as a substrate in enzymatic reactions (Jomova & Valko, 2011). GSH is a unique barrier for scavenging of hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot-}$) and hydroxyl ($HO^{\cdot}$) species (Misak et al., 2018). Once GSH levels are decreased, lipid structure can be affected via the initiation of LPO. In this current study, administration of Indo caused GSH diminishment and in turn LPO elevation. These alterations can be explained with the free radical formation potential of Indo on different parts of the gastrointestinal system. One of these parts that is affected by Indo is the stomach. Ugan & Un (2020) reported the same alterations in GSH and LPO levels in their study, which emphasises the deleterious effects of Indo in this tissue. Our results for LPO are also in line with previous reports (Varghese, Faith, & Jacob, 2009; Moustafa et al., 2013). PC can be considered products of the peroxidation process, which harms both the lipid and protein structure of membranes (Sivalingam et al., 2007). Indo caused an elevation of PC levels of Indo-treated tongues, perhaps the same way as with the alteration of GSH and LPO levels. This elevation is also evidence for increased oxidative stress caused by Indo. The present results are in accordance with reports by Banerjee, Maity, Nag, Bandyopadhyay, & Chattopadhyay (2008). The administration of the antioxidant combination reversed the levels of these parameters in the Indo group. Vit C protects GSH from further oxidation, and helps in the stabilization of the GSH/GSSG ratio. Vit E aids the regeneration of Vit C, and also protects the membrane structure from



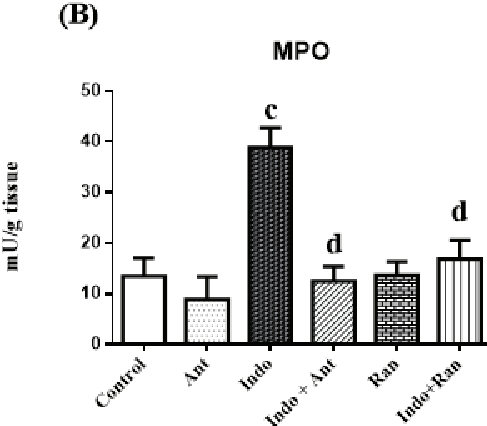


Figure 3. The tongue tissue LDH (A) and MPO (B) activities of control and experimental groups. Values are expressed as mean ± SD. [a]P<0.001 vs control group, [b]P<0.05 vs Indo group, [c]P<0.0001 vs control group, [d]P<0.0001 vs Indo group, Ant: antioxidants (vitamins-selenium); Indo: indomethacin; LDH: lactate dehydrogenase; MPO: myeloperoxidase; Ran: ranitidine; SD: standard deviation.

DISCUSSION

The tongue encounters most of the blood supply in the course of the feeding process, when considering its skeletal muscle structure, taste sensing functions and connections with nerve activity (Toda, Ayajiki, & Okamura, 2012). Unfortunately, it is inevitable that the tongue tissue will encounter free radical circulation when various agents like ethanol or some NSAIDs are used. These agents have recently been implicated in causing the elevation of free radicals, which ultimately harm the tongue tissue (Baek et al., 2019; Bayrak et al., 2021).

Indo has been shown to be a potent pro-oxidant initiator (Nagano et al., 2005). In addition, the drug affects mitochondrial function like all NSAIDs (Bindu et al., 2020). In different tissues, Indo's many free radical production mechanisms exist. One of the mechanisms that affects this function is the inhibitor effect of Indo on gastric peroxidase (Chattopadhyay, Bandyopadhyay, Biswas, Maity, & Banerjee, 2006). This drug causes aconitase inhibition in mitochondria. This leads to the release of free iron following inhibition and decomposition of the aconitase structure, which is stabilized by iron-sulphur interaction. On account of this free iron, hydroxyl radical generation starts (Maity et al., 2009). In addition, studies by Turkyilmaz et al. (2019a and 2019b) on the effect of Indo on gastric and intestinal damage indicates that Indo inhibits intestinal epithelial cells by dramatically decreasing cell proliferation, and also interferes with caspase 3, 8 and 9 expressions in gastric media. The elevation of caspase 8 expression, as well as the diminishment of

unwanted LPO (Ali, Ahsan, Zia, Siddiqui, & Khan, 2020). Beta carotene also supports lipid protection (Ali et al., 2020), while Se as selenoprotein can diminish ROS production (Cai et al., 2019). Ching, Haenen & Bast (1993) proved that H_2-blockers like Ran and cimetidine had powerful radical scavenging activity. Based on this approach, we can assume that Ran might have increased GSH levels, and as well decreased LPO and PC levels due to its proven antioxidant effect.

Oxygen is necessary for the progression of life processes. Mitochondria utilise oxygen with a high ratio during aerobic reactions, however, a small percent of the oxygen is transformed to $O_2^{\cdot-}$ (Assi, 2017). Although this function is normal for cells, elevation of this situation leads to the excessive formation of radical molecules including $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$ (Kajarabille & Latunde-Dada, 2019). In tongue tissues of Indo treated rats, lessened activities of GPx, SOD, CAT and GST (enzymatic defence system against ROS) were observed. Similarly, decreased antioxidant enzyme activities were reported in Indo-induced organ damage and ethanol-induced tongue injury by Abdallah, (2010), Koriem, Gad, & Nasiry (2015), Atalay et al., (2016) and Bayrak et al. (2021), respectively. The diminishments of GSH levels may be as a result of its excessive depletion, which thereafter also affects GSH-dependent enzymes like GPx and GST. GPx is also a selenoprotein, capable of directly scavenging H_2O_2 (Guillin et al., 2019). GST employs GSH as a co-substrate for detoxifying harmful substances. SOD transforms $O_2^{\cdot-}$ to H_2O_2, a substrate for both CAT and GPx (Ali et al., 2020). The vitamin combination, including Se and Ran, increased the activities of the aforementioned enzymes in the Indo group. This ameliorating effect can be associated with the existence of Se for stabilizing GPx activity. In addition, Vit E is also capable of scavenging a large scale of radical molecules (from peroxides and superoxides derived by xanthine), while Vit C helps in its regeneration (Miyazawa, Burdeos, Itaya, Nakagawa, & Miyazawa, 2019). Beta carotene and Ran may have also decreased radical substances by stabilizing GSH levels and ameliorating antioxidant enzyme activities in the tongue.

LDH is an important enzyme for carbohydrate metabolism. Its elevated activities can be considered proof of possible oxidative damage. Indo has been reported to cause changes in energy metabolism. It affects mitochondrial functions by altering energy flux from aerobic to anaerobic glycolysis (Khan, Yusufi, & Yusufi, 2019). By considering this theory and the formation of the tongue structure as resembling skeletal muscle, the present results suggest that LDH elevation in the Indo treated group is connected with oxidative damage. Moreover, Pawar, Anap, Ghodasara & Kuchekar (2011) reported elevated serum LDH activities in Indo-induced enterocolitis via emphasising the inhibition of COX and prostaglandins by Indo. Ben Amara et al. (2011) reported that the ameliorating effect of Vit E and Se on LDH enzyme activities might be related to the antiradical/antioxidant activities of the vitamin. H_2-receptor antagonists like cimetidine and Ran were also reported to have an inhibitory effect on LDH, although Ran was less inhibitory than cimetidine (Gill, Sanyal, & Sareen, 1991). The outcome of the present finding is in agreement with the aforementioned reports. Hence, the combination of vitamins, Se, and Ran successfully decreased LDH activity in Indo-induced tongue injury.

MPO (an important constituent of neutrophils) is released when the infiltration of leukocytes occurs. Through this approach, Indo has been proven to increase MPO activities in gastric tissues (de Araújo et al., 2018), and as well cause inflammatory reactions and epithelial losses in small intestinal tissues (Yamamoto, Itoh, Nasu, & Nishida 2014). Besides, Indo has been declared as having an elevator effect on MPO-associated neutrophil infiltration in Indo-induced ulcer models (Abdel-Raheem, 2010). In addition, some NSAIDs have been reported to be targets of mitochondrial electron transport system (ETC) complex I, thereby increasing mitochondrial oxidative stress (Bindu et al., 2020). Naturally, this is an indicator for increased H_2O_2 levels as parallel to elevated oxidative stress. In this study, tongue MPO activities of the group to which Indo was administered were found to be increased. Vitamin combination with Se may have decreased this activity in the group via radical scavenging effect. Ran was also proven to decrease excess MPO activities by reducing neutrophil infiltration in gastric tissue. The present findings for Ran are in accordance with reports by de Araújo et al. (2018).

CONCLUSION

According to the results, Indo caused an elevation in oxidative stress in the tongue tissue of rats. We may conclude that the protective effects of vitamin-Se treatments and Ran administrations on this injury either using their antioxidative properties or their positive effects on the regulation of energy metabolism.

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Original Article

Validation and reliability of the Turkish version of the student satisfaction and self-confidence in learning scale for clinical pharmacy education

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ABSTRACT

Background and Aims: Innovations in clinical pharmacy education that are supported by simulations can improve student knowledge and skills. The aim of this study is to assess the validity and reliability of the Turkish version of the Student Satisfaction and Self-Confidence in Learning Scale (SCLS) among pharmacy students in Türkiye.
Methods: This study was conducted at clinical pharmacy departments of Ankara University and Altinbas University between March 28-April 28, 2022. Students taking an online simulation-based learning class during their clinical pharmacy education were eligible. The study adapts the SCLS as developed by the National League for Nursing (NLN, 2004) for pharmacy students, then translates it into Turkish and evaluates the inter-rater and test-retest reliabilities.
Results: A total of 176 students participated in the study, the majority of whom are female (76.1%); the participants have a mean ± standard deviation (SD) age of 23.42±2.82 years. The coefficients for the inter-rater and test-retest reliabilities were found to be high for the scale ($r=0.953$, $p<0.05$; $r=1$, $p<0.05$, respectively), with Cronbach's alpha being calculated as 0.95.
Conclusion: The Turkish version of the SCLS is concluded to be a valid and reliable tool for pharmacy students receiving clinical pharmacy education.

Keywords: Clinical pharmacy, Simulation-based learning, Validation, Reliability

INTRODUCTION

Clinical pharmacy education advances students' pharmacotherapy, pharmaceutical care, and patient-centered knowledge and skills through the use of innovative educational approaches (Seybert et al., 2019). Innovations in clinical pharmacy education that are supported by simulations, games, and virtual reality are able to improve students' skills regarding patient-centered care, medical history checks, and counseling practice with standardized patients, as well as interprofessional activities using simulated mannequins by imitating the real world (Seybert et al., 2019; Lynch, Griffin, & Vest, 2018). Such innovations meet the growing demand for impact and creativity academicians in the field of clinical pharmacy seek (Seybert et al., 2019).

The Accreditation Council for Pharmacy Education (ACPE, 2007) describes pharmaceutical simulations as activities or events that repeat practices from the pharmaceutical environment. It is also suggested that pharmacy educators use innovative educational

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technologies and techniques, including a variety of teaching and evaluation methods (ACPE, 2007). These technologies and techniques complement the use of simulations for assisting students in developing critical thinking and problem-solving skills. Simulation-based learning (SBL) allows students to practice their knowledge and skills in a safe and controlled environment (Steadman et al., 2006) and is beneficial in its standardization and repetition of content, interactive learning in a clinical setting without risk to patients, and its ability to design goal-oriented clinical experiences. SBL employs a wide range of simulation environments, ranging from standard to high-quality patient simulations, such as human patient simulators (high, medium, or low-quality patient simulators/dummies), partial task trainers, standardized patients, virtual reality simulation, screen-based computer simulators, and integrated simulators (Steadman et al., 2006; Korayem & Alboghdadly, 2020). Simulation training in advanced clinical pharmacy education has been shown to improve students' confidence, basic knowledge, and communication skills, to encourage critical thinking, and to strengthen patient care (Seybert et al., 2019).

SBL is used in various health disciplines such as medicine, nursing and pharmacy (Korayem & Alboghdadly, 2020). Although SBL has been applied in military, medical, and nursing programs for many years, the use of fidelity human patient simulations in clinical pharmacy education didn't occur until 2006 (Seybert et al., 2019), and pharmacy faculties and health care institutions have adopted SBL in various clinical education formats for all levels of learners and practitioners. Nowadays, many research articles from all around the world have studied how to incorporate SBL into clinical pharmacy education and SBL outcomes (Ferrone, Kebodeaux, & Fitzgerald, 2017; Gustafsson, Englund, & Gallego, 2017; Mak, Fitzgerald, & Holle, 2021). However, only a limited number of faculties have incorporated SBL in Türkiye, and no validated or reliable survey exists in the pharmacy field in Türkiye for measuring the outcomes of SBL in clinical pharmacy education. Surveys have been developed for use in the nursing and medical fields (National League for Nursing, 2006; Turatsinze, Willson, & Sessions, 2020). Therefore, this study aims to evaluate the validation and reliability of the Turkish version of the Student Satisfaction and Self-Confidence in Learning Scale (SCLS) developed by NLN (2004) among pharmacy students.

MATERIALS AND METHODS

Study population and settings

This study was conducted at the clinical pharmacy departments of Ankara University and Altinbas University between March 28-April 28, 2022. Students who were enrolled in an online simulation-based class for learning clinical pharmacy were invited to participate in the study. The SBL activity was carried out using the Monash University-developed program MyDispense®, which was adapted to simulate the dispensing method in Türkiye by the Clinical Pharmacy Department of Altinbas University. During the online SBL, students were given a prescription for chronic diseases such as diabetes mellitus, hypertension, and asthma. The students were responsible for dispensing medications while asking appropriate questions and providing patient counseling. Although the same program was used, the two universities used different instructors and different medicines in the prescriptions.

To assess the students' satisfaction and self-confidence with SBL for clinical pharmacy education, a search was made in the literature for an appropriate scale, and the authors decided to use the SCLS (NLN, 2004). SCLS is used to measure students' satisfaction and self-confidence levels regarding their clinical skills and care as well as in relation to the instructions, materials, and instructor (NLN, 2006). The scale contains 13 questions that are scored using a 5-point Likert-type scale (0 = totally disagree to 4 = totally agree). The SCLS is a validated survey for SBL and has a Cronbach alpha of 0.94.

The sample size of the study population was determined based on the recommendations for reporting the results of studies on instrument and scale development and testing (Streiner & Kottner, 2014; Streiner, Norman, & Cairney, 2015). The minimum sample size for the study was calculated as 130 participants. This study was approved by the Ethical Committee of Ankara University (Approval no: 05-57, date: 07.02.2022). The informed consent was obtained from the students before collecting survey responses.

Translation process

Before translating the SCLS, permission was obtained from NLN, after which the survey was then translated from English to Turkish by a fluent English-speaking investigator (AS). Then for the back translation, another fluent English-speaking investigator (NA) translated the SCLS back from Turkish into English. Both translations were reviewed by another independent investigator (AB), and the Turkish version of the SCLS was developed. After this, the investigators analyzed the most recent Turkish translation of the SCLS for cultural and conceptual content and equivalence in addition to grammar for particular use in clinical pharmacy education.

Validation process

The students were asked to complete the survey twice (test-retest with a 15-day time interval). The survey was implemented online using Google Forms, and the students were required to answer all the questions in order to complete the survey. The students who only completed the survey once had their responses excluded.

The questioner then evaluated the SCLS for its internal reliability and validation by measuring Cronbach's alpha of reliability and intraclass correlation as presented in the statistical analysis.

Statistical analysis

The data entry and analyses required the use of the package program SPSS (version 26). Descriptive statistics were presented as mean ± *SD*, while categorical variables were presented as percentages. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. Test-retest reliability (the baseline and post-15-days retest) and inter-rater reliability were evaluated using the intraclass correlation coefficient. The internal reliability estimates were calculated using Cronbach's alpha of reliability.

RESULTS

A total of 176 pharmacy students participated in the study with a mean ± standard deviation (SD) age of 23.42 ± 2.82 and 54.5% being from Ankara University. The mean age was found to be 23.42 ± 2.82 years. The distribution of students in terms of year of study showed 35.2% to be 3rd-year students, 60.3% to be 4th-year, and 4.5% to be 5th-year. The majority of the student participants are female (76.1%; Table 1).

According to the students' pretest responses to the SCLS, Table 2 shows most of the students agreed or strongly agreed with the following survey items: Item 3 (Whether the student enjoyed on how the instructor taught the simulation; 83.5%), Item 5 (Whether the instructor used suitable way; 80.2%), Item 9 (Whether the instructors used helpful resources; 84.7%), Item 10 (Whether the students knew their responsibility; 83.0%) and Item 13 (Whether the students' knew the instructor's responsibility; 84.7%).

Table 1. Characteristics of the Students who Participated in the Study (*n* = 176).

Variables	*n* (%)
Gender	
Male	42 (23.9)
Female	134 (76.1)
Age	
Mean ± SD	23.42 ± 2.82
School year	
Third year	62 (35.2)
Fourth year	106 (60.3)
Fifth year	8 (4.5)
University	
Altinbas University	80 (45.5)
Ankara University	96 (54.5)

Table 2 shows most of the students agreed or strongly agreed with the following survey items for the posttest: Item 2 (about the variety of materials and activities provided by simulation method; 80.2%), Item 4 (about student motivation about teaching materials for the simulation ; 81.2%), Item 9 (about helpfulness of the resources provided by the instructor; 81.3%), and Item 13 (about instructor responsibility to tell what students need to learn; 82.3%).

Table 3 displays the item-total correlation, Cronbach's alpha., and intraclass correlation. Values for SCLS' subscales. The item-total correlation analysis revealed all items to have a total correlation value greater than 0.75. Cronbach's alpha for the 13-item scale was calculated as 0.95. The study also found the intraclass correlation and test-retest reliability values to be high for the SCLS ($r = 0.952$, $p < 0.001$; $r = 0.706$, $p < 0.001$, respectively; Table 3).

DISCUSSION

This study has demonstrated high reliability for the Turkish version of the SCLS with a Cronbach's alpha of 0.95, which is higher when compared to the original scale (α = 0.94). The Turkish version of the SCLS can be used to assess students' satisfaction and self-confidence regarding simulation-based learning in clinical pharmacy education. Another study was conducted for adapting the SCLS to Turkish, except it involved simulation education among nursing students (Karacay & Kaya, 2017); their study achieved a Cronbach's alpha of 0.88 and removed Item 13 to improve the alpha to 0.90. Their results resembled those

Table 2. Students' Responses to the Scale Items.

Survey item numbers and their contents*		Agreement	
		Pretest	Posttest
Item 1.	Helpfulness of the teaching via simulation method	79.0%	79.5%
Item 2.	Variety of materials and activities provided by simulation method	79.6%	80.2%
Item 3.	Student enjoyment on how the instructor taught the simulation	83.5%	78.4%
Item 4.	Student motivation about teaching materials for the simulation	77.3%	81.2%
Item 5.	Suitability of the teaching by the instructor	80.2%	79.5%
Item 6.	Student confidence regarding mastering the simulation	71.6%	75.1%
Item 7.	Student confidence regarding simulation covers critical content	73.3%	78.4%
Item 8.	Student confidence regarding developing skills/obtaining knowledge	71.1%	73.9%
Item 9.	Helpfulness of the resources provided by the instructor	84.7%	81.3%
Item 10.	Student responsibility for learning what they need to know	83.0%	77.8%
Item 11.	Student knowledge on how to get help when they don't understand	77.8%	76.7%
Item 12.	Student knowledge on how to use the simulation to learn critical aspects	69.9%	74.4%
Item 13.	Instructor responsibility to tell what students need to learn	84.7%	82.3%

*The survey verbatim can be obtained from NLN via permission. The content of the items were modified to give an idea to the readers.

Table 3. The Inter-Rater and Test–Retest Reliability of the Scale Items.

Survey item numbers and their contents*		Cronbach's alpha**
Item 1.	Helpfulness of the teaching via simulation method	0.837
Item 2.	Variety of materials and activities provided by simulation method	0.845
Item 3.	Student enjoyment on how the instructor taught the simulation	0.801
Item 4.	Student motivation about teaching materials for the simulation	0.864
Item 5.	Suitability of the teaching by the instructor	0.802
Item 6.	Student confidence regarding mastering the simulation	0.947
Item 7.	Student confidence regarding simulation covers critical content	0.821
Item 8.	Student confidence regarding developing skills/obtaining knowledge	0.846
Item 9.	Helpfulness of the resources provided by the instructor	0.786
Item 10.	Student responsibility for learning what they need to know	0.792
Item 11.	Student knowledge on how to get help when they don't understand	0.858
Item 12.	Student knowledge on how to use the simulation to learn critical aspects	0.829
Item 13.	Instructor responsibility to tell what students need to learn	0.753
Cronbach's alpha for the overall scale		0.953
Intraclass correlation for the overall scale		0.952
Test-retest reliability		0.706

*The survey verbatim can be obtained from NLN via permission. The content of the items were modified to give an idea to the readers.
**This table shows the intraclass correlation values (Spearman's rho; $p < 0.001$), all of which are statistically significant.

from the current study. Another study measured the Turkish validity and reliability of the SCLS among nursing students and calculated Cronbach's alpha values between 0.77–0.85 (Unver et al., 2017), which are lower than what the current study found.

Various evaluations on testing the validation and reliability of the SCLS have been done in other countries. The Chinese version (Chan, Fong, & Tang, 2015) of both SCLS' satisfaction and self-confidence subscale components among nurses showed high reliabilities, with Cronbach alphas of 0.95 and 0.97, respectively. The Spanish version (Farrés-Tarafa et al., 2021) of the SCLS demonstrated high internal consistency and reliability for the overall scale as well as for each of its components, with a Cronbach's alpha of 0.88 for each of the components. The Arabic version (Grande et al., 2022) of the SCLS showed an alpha value greater than 0.70 among nursing students. Lastly, the Portuguese version (Almeida et al., 2015) of the SCLS revealed a Cronbach's alpha of 0.86 among nurses. In this sense, the Turkish version appears to have one of the highest reliability values. Moreover, this was the first study to evaluate the validity and reliability of the SCLS among pharmacy students.

Overall, the students expressed positive responses to the questions regarding their satisfaction and self-confidence. This resembles another study conducted in Türkiye that evaluated the satisfaction, confidence and motivation, clinical experience, and decision-making among 81 pharmacy students (Aksoy & Ozturk, 2021). In Australia, pharmacy students involved in SBL agreed or strongly agreed that they had been provided a learning opportunity to safely make errors knowing that patients could not be harmed and that the learning was more realistic than addressing a similar patient case on paper (Mak et al., 2021). In the United States of America, students had favorable perceptions about the SBL activity, and their knowledge was significantly improved (Rude et al., 2022).

Pharmacy education must be developed, revised, or updated in order to meet the standards for providing patient-centered care in an interdisciplinary environment and to have effective communication with other health care professionals and patients (Kayyali et al., 2019). Although workplace learning is the gold standard for training pharmacy students to fulfill the standards, obtain funding, and find workplaces is a daunting task for faculties. Therefore, simulating workplaces by using SBL is an effective technique for training students and must be adopted by faculties in Türkiye. Only three out of 60 pharmacy schools in Türkiye use SBL in their clinical pharmacy education. More encouragement and good practice examples in teaching and learning must be provided for those who have yet to implement SBL.

This study marks the first time that students from Ankara University had participated in an online SBL activity. The results from the survey show positive reflections that indicate the country should continue integrating these innovative approaches in clinical pharmacy education.

While implementing a new innovative educational approach, the instructors faced certain challenges. The first challenge was the process obstacles. Students were required to read pre-class materials prior to participating in the online SBL course. Students who did not read the pre-class materials were unable to finish the SBL activity on time. The second challenge was the facility obstacles. Students were required to bring their own

electronic devices or sit in the computer laboratory to access the online SBL course because the computer laboratory had limited capacity. The last challenge was the cultural obstacles. Some students were reluctant to use technology or innovative approaches in learning, which thus negatively affected instructors' ability to teach SBL.

This study has some limitations. By having been conducted in two different settings, the lecture contents and instructors likely had slight differences. This has the ability to affect how students responded to the survey questions. However, the pretest and posttest responses were specific to each student and did not affect the validation process. This study aimed to evaluate the validity of the current Turkish version of the SCLS, so the findings of the study have not come from an observational study. Also, the response rates were not calculated, and the number of participants was more than what had been calculated for the sample size.

In conclusion, the Turkish version of the SCLS is a valid and reliable tool for use with pharmacy students receiving clinical pharmacy education. This version can be widely used to assess the satisfaction and self-confidence of pharmacy students who take online simulation-based learning activity in their clinical pharmacy education.

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Original Article

Community pharmacists' epilepsy counseling: A theory of planned behavior approach

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ABSTRACT

Background and Aims: Community pharmacists play a major role in healthcare. To improve the success in epilepsy treatment, community pharmacists' involvement is important. This study aimed to express the influencing factors of community pharmacists' counseling to patients with epilepsy.
Methods: Semi-structured interviews were held with fifteen community pharmacists in the Theory of Planned Behavior framework. A directed content approach was performed to analyze the study results.
Results: The participants emphasized that easy access to community pharmacists is worthy. Patients with epilepsy have various expectations from pharmacists. However, lack of communication was determined between community pharmacists and physicians. Irregular drug usage and getting inadequate and misleading patient information are highlighted as perceived major barriers/difficulties. All pharmacists have the intention to receive training on epilepsy.
Conclusion: Improving communication between pharmacists and physicians may help to enhance collaborative work in epilepsy management and to increase treatment success. Moreover, vocational training may be organized to enhance pharmacists' counseling.

Keywords: Community pharmacists, counseling, epilepsy, theory of planned behavior

INTRODUCTION

Epilepsy is a neurological disease that affects approximately 50 million people around the world (World Health Organization, 2019). Comorbidities such as depression, anxiety, migraine, and stroke are more common in patients with epilepsy (PWE) (Selassie et al.,2014; Keezer, Sisodiya, & Sander, 2016), and drug interaction risk is high between antiepileptics and other drugs (Bosak, Słowik, Iwańska, Lipińska, & Turaj, 2019). Epilepsy treatment may also include polytherapy to keep the disease under control (Park, Kim, & Lee, 2019). Adverse drug effects in PWE decrease the quality of life (Perucca & Gilliam, 2012; Micoulaud-Franchi, Bartolomei, Duncan, & McGonigal, 2017). Additionally, medication adherence is a substantial factor in the success of epilepsy management; however, patients are concerned about using these medicines and have poor adherence (Dayapoğlu, Turan, & Özer, 2021).

Previous studies indicate that pharmacists have various contributions to epilepsy management, such as drug-related issues, adherence, and education of patients (Koshy, 2012; Reis, Campos, Nagai, & Pereira, 2016). McAuley, Miller, Klatte, & Shneker (2009) exhibited that PWE desire to discuss more drug-related problems with their community pharmacists. Eshiet, Okonta, & Ukwe (2021) reported pharmacists' counseling improves the quality of life in epilepsy. Another point is that unmet healthcare needs are more common in PWE (Mahendran, Speechley, & Widjaja, 2017). In this context, PWE need more pharmacist counseling, and the pharmacist's role for patients with epilepsy and/or their relatives becomes important in epilepsy management. This study aims to put

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forth the factors that influence community pharmacists while counseling patients with epilepsy in the light of the Theory of Planned Behavior (TPB). To the best of the author's knowledge, this is the first study exploring the influencers of pharmacists' counseling to PWE in a qualitative approach based on TPB.

MATERIALS AND METHODS

Theoretical framework

In various studies, the TPB model has been used to examine the counseling of health professionals (Amin & Chewning, 2016; Lin, Fung, Nikoobakht, Burri, & Pakpour, 2017), and to predict the factors influencing pharmaceutical care (Rawy, Look, Amin, & Chewning, 2021). According to the TPB approach, attitude, subjective norms, and perceived behavioral control influence the intention of an individual to perform a behavior (Ajzen, 1991). The TPB framework has also been applied in studies related to epilepsy such as medication adherence in PWE (Lin, Updegraff, & Pakpour, 2016) and neurologists' attitudes, and barriers toward epilepsy surgery (Erba, Moja, Beghi, Messina, & Pupillo, 2012).

Study design and data collection

Izmir Katip Çelebi University Social Research Ethics Committee (No: 2020/09-04) approved the study. Fifteen interviews were performed in February 2021. The Consolidated Criteria for Reporting Qualitative Research (COREQ) was used for reporting (Tong, Sainsbury, & Craig, 2007). The interviews were conducted and transcribed verbatim by the researcher who has experience in qualitative research and community pharmacy and a Ph.D. degree.

The interview guide was constructed according to the TPB framework (Francis et al., 2004; Fishbein & Ajzen, 2010). The interview guide has seven questions, including attitude (importance/advantages of pharmacists counseling to PWE; factors affecting the successful management of epilepsy), subjective norms (approaches/expectations of physicians and PWE/patient relatives about pharmacist consultation), perceived behavioral control (difficulties/barriers while counseling PWE/patient relatives; any differences in giving counseling to PWE compared to other patients) constructs, and a question about willingness to take a vocational training, course, etc. on epilepsy in the future.

Purposive and snowball sampling methods were used. Attention was paid to balancing the demographics of pharmacists. Semi-structured interviews were held in Turkish and lasted approximately 15 minutes. None of the interviews were repeated. Community pharmacists practicing in Turkiye were informed about the study, and those who volunteered to participate indicated their available times. The interviews were held by calling the pharmacists at that time frame, and informed consent was obtained. The interviews were audio-recorded in a quiet environment with attention to privacy.

Data analysis

Data analysis was performed via directed content approach within the TPB framework. A directed content analysis uses a theory or research results for guidance and aims to validate or expand the theory or framework (Hsieh & Shannon, 2005). The initial codes were identified according to the constructs of TPB. Interview data, English transcripts, and codes were checked several times for accuracy.

RESULTS

The demographic data are shown in Table 1. Eight female and seven male community pharmacists participated in the study. The experience in the profession ranges from 2 to 28 years. The majority of the participants have been working as a pharmacist for more than ten years. The pharmacies' locations were classified into three groups, as presented in Table 1.

Table 1. Demographic information of the participants

	n
Gender	
Female	8
Male	7
Experience in profession (year)	
<10	4
10-20	7
>20	4
Pharmacy location	
Neighborhood pharmacy	5
On-street	4
Near health center	6

Behavioral Beliefs/Attitudes

Most pharmacists (n=11) highlighted that informing patients, particularly on the regular use of epilepsy medications, contributes to controlling the disease.

P3: *"Since regular use of medications by patients with epilepsy will prevent them from having seizures or other complications, in this sense, providing information to patients with epilepsy and telling them about their medication are essential for patient adherence."*

P5: *"Epilepsy medicines are specific medicines that require patients' 100% adherence with the treatment program. To achieve this adherence, the right explanation of the medicine, the right explanation of its use, what can happen when the missed dose is taken, or the treatment is interrupted without consulting the physician and pharmacist, that is to say, informing what kind of problems can happen shows the importance of pharmacist consultation here."*

Four pharmacists expressed that PWE can consult community pharmacists easily. Two of them remarked,

P8: *"Pharmacies are considered to be primary health care institutions. And they (patients) see pharmacies as the first place they visit outside the hospital and where they receive consultancy ser-*

vices. And I think this makes a greater sense, especially in small places. ….they see it as a place where they can ask questions and get information more comfortably in a more friendly environment than that of a physician. Therefore, I think the counseling service that the pharmacy can provide is important for the patients."

P9: *"…Our physicians are very busy ….in that sense, they (patients) sometimes do not have the opportunity to ask what they want. In general, these patients try to find out the details of their diseases from the pharmacist. At this point, I think it is vital."*

The majority of pharmacists (n=13) indicated the importance of patient medication adherence. Pharmacists cited (n= 10) that the contribution of the relatives/family in epilepsy is noteworthy. A pharmacist noted,

P6: *"It is necessary to ensure that medication adherence is complete, and in my opinion, it is necessary to try to provide familial support. In my opinion, the most important factor in the success of treatment is the proper, regular use of drugs with the correct dosage. This is the critical point of treatment. Family support is needed in this regard…"*

Normative Beliefs/Subjective Norms

Two pharmacists stated that patients/their relatives do not have many expectations of them. A pharmacist attributed this to the fact that epilepsy requires expertise. The pharmacist indicated,

P8: *"Epilepsy disease, medicines used in epilepsy disease, treatment of epilepsy disease is a subject that requires specialty. I guess the relatives of the patients see this with this eye, and I think they do not expect much consultancy service from the pharmacy. Because, until now, for example, apart from taking epilepsy medicines, we have not had a patient expectation or asking any information from us about his illness or medicines."*

The rest of the pharmacists discussed that patients ask for almost everything about medicine. Besides, they need support about life challenges. A pharmacist shared,

P11: *"…For example, when he/she goes to his/her physician and changes the dose, he/she comes to me and expects a lot while taking a medicine… For example, I realize that it is very important for the patient to be able to contact me when he/she has a problem with medication. …even though things like this happen, for example, they feel the need to get support for the difficulties in the patient's life…"*

In addition, it is emphasized that infant and children relatives ask mainly about the dosage of the medicine, particularly the dosage of medications in syrup forms. A pharmacist indicates,

P14: *"In pediatric patients, the dosage can change constantly… Because as the child grows, he/she may need to take a different dosage every year. It needs to be more tightly controlled."*

Seven pharmacists shared they do not have contact with physicians about epilepsy treatment. A pharmacist expressed, *"I mean, frankly, we've never had any contact with a physician about it until today."* Other pharmacists stated the expectations of physicians as informing patients about drug usage, drug interactions, and adverse effects. A pharmacist shared,

P11: *"I think it may be to provide detailed information about the drug's usage, side effects, and follow it up. As I said, because physicians are very busy, unfortunately, they cannot follow up with each patient for the slightest dose change or the start of a new drug, especially in public hospitals, and I think they may be waiting for us to follow up."*

Control Beliefs/ Perceived Behavioral Control

The pharmacists complained of several difficulties/barriers. Eight pharmacists cited irregular drug usage, and four pharmacists were concerned about getting inadequate and misleading information from patients/relatives.

P9: *"So once we had a patient who fell to the ground and hit his head and bled while having a seizure. We tried to pay close attention, he fell very close to the pharmacy. We ask "if you are missing the dose?", he says "no, I am taking it on time". But in the SSI (social security insurance) system, we see when the drugs should start and end, from the number of boxes and tablets or daily doses. You see that the patient's drug expiration time does not end on time, which means that he does not use it regularly. But when you ask, he doesn't give you information, he says no, he says that he uses his medicine regularly."*

Four pharmacists identified generic drug substitution as an arduous process in PWE. A pharmacist indicated,

P2: *"If the medicine is not on the market, the patient is in trouble. And he/she doesn't want a generic. For example, when an antibiotic is offered as a generic, it is not very important for him/her, but if it is an antiepileptic drug, then it becomes very difficult for him/her to accept even if you offer a generic."*

Six pharmacists stated that there was no difference when counseling epilepsy patients compared to other patients. The rest of the participants pointed out some differences. A pharmacist remarked on the necessity of being more sensitive toward PWE.

P11: *"I think there are differences. I think it is a disease that needs to be treated more sensitively. In other words, it is a process that requires a more detailed and healthier follow-up of consultancy… let's take a hypertension patient as an example, it is also a chronic disease, but after starting a medication and giving counseling, we see that it is very likely that the patient does not have a problem and adapts to the medicine, and maybe using it for years… But epilepsy patients are more sensitive. Maybe they also need more counseling because they don't know."*

Another pharmacist underlined the bashfulness of PWE.

P9: *"That is to say, other patients express their illness more clearly. For example, a patient with coronary artery disease is not ashamed of his/her illness when he/she tells about it or his/her life… but in epilepsy patients, abashment, embarrassment, hiding, abstaining from telling much are prevalent. That's why you have a little more difficulty because while questioning the patient, is he/she telling the truth, or is he/she hiding something? He/She says "I'm taking my medicine", but you look through the system, it (the medicine) doesn't actually end in the time it should..."*

Lastly, all pharmacists expressed their intention and desire to increase their knowledge about epilepsy in the future. It was underlined that epilepsy is a specific area, they have limited knowledge, and it needs to be updated. Two of the participants express,

P8: *"I think it is one of the areas where pharmacists are lacking, that is, one of the aspects lacking in terms of disease and drug information. But of course, these can be solved with vocational training."*

P15: *"Ever since I opened my pharmacy, I have realized that we do not have enough knowledge. We need to update it constantly."*

DISCUSSION

Pharmacists cited that ensuring regular usage of medications and patients' easy access to community pharmacists are substantial for epilepsy treatment. Additionally, according to pharmacists, patient adherence and support from family members/relatives influence the success of the treatment. The vast majority of the pharmacists indicated they are asked for almost everything, even for support in life challenges. Nearly half of the participants expressed that they are not in contact with physicians. Irregular drug usage, lack of and misleading information are cited as major difficulties/barriers in the consultation process. All pharmacists are willing to improve themselves with training in the future. Examining the factors influencing pharmacists' counseling on this issue will help expand the counseling process and healthcare services for PWE provided by community pharmacies.

Existing literature indicates that family support improves antiepileptic medication adherence (Yang, Hao, Yu, Xu, & Zhang, 2018). In line with this, the participants mainly underlined the necessity of adherence and the contribution of family/relatives.

Ma et al. (2019) exhibited that pharmacists positively impact medication adherence in pediatric PWE. Similarly, in the current study, the pharmacists expressed that many questions about pediatric dosage forms were asked.

In the current study, the pharmacists highlighted the bashfulness of PWE, and not being sufficiently informed or being misinformed complicates the counseling process. This situation may occur due to the social environment that PWE are faced with. PWE are exposed to social exclusion and discrimination (Shi, Liu, Wang, Li, & Zhang, 2021), and stigmatization in epilepsy is much more than other neurological diseases (Ak, Atakli, Yuksel, Guveli, & Sari, 2015). Besides, it is stated that PWE generally prefer a concealment strategy to deal with distressing situations in their lives (Shi et al., 2021). To provide effective counseling, it is necessary to ensure that patients give accurate and sufficient information about their treatment without hesitation. Moreover, psychological situations related to stigmatization, which is intensely seen in PWE, reduce help-seeking behaviors (Shi et al., 2021). As mentioned in the current study, the advantage of easy access to community pharmacies and pharmacists' sensitive behaviors may encourage patients to collaborate with pharmacists for epilepsy management. In addition, pharmacists indicated that patients have many expectations for almost everything related to medicines and support in life challenges. In the literature, availability and accessibility issues are expressed to be the two of the reasons for the unmet healthcare needs of PWE (Mahendran et al., 2017). By improving pharmacists' counseling behaviors and expanding their roles in epilepsy treatment, community pharmacies may play a central role and help to decrease the unmet healthcare needs of PWE. According to the findings of global research, there were diverse barriers to the generic substitution of antiseizure medications (Niyongere et al., 2022). Similarly, in the present study, the participants mentioned that the generic substitution of antiepileptics is undesired by PWE. Berg, Gross, Haskins, Zingaro, & Tomaszewski (2008) surveyed physicians and patients on this issue and concluded that they have efficacy concerns and relate breakthrough seizures with the generic substitution of antiepileptics.

Reducing communication gaps between healthcare providers is substantial for patient care (Amin & McKeirnan, 2022). It is a remarkable result that nearly half of the pharmacists stated that they had no communication with physicians. Parallel with this, from the physicians' perspective, insufficient communication was one of the barriers to building effective collaboration with pharmacists (Amin & McKeirnan, 2022). The communication and collaboration between physicians and pharmacists need to be improved.

The current study findings demonstrated that pharmacists are not sufficiently knowledgeable, and they all intend to enhance their knowledge on this issue. Parallel to this finding, in a study, community pharmacists expressed that they are not much more experienced with epilepsy and require more training (Bacci, Zaraa, Stergachis, Simic, & White, 2021). Ngoh (2009) reported that pharmacists could cope with patients' poor health literacy and improve medication adherence by collaborating with patients and healthcare professionals. Improving community pharmacists' knowledge will contribute to health outcomes. Pharmacists intend to promote their knowledge and skills in the management of epilepsy. The involvement of community pharmacists in epilepsy treatment in communication with physicians could reduce the burden on physicians and hospitals and facilitate the patient's access to health services.

CONCLUSION

The findings of this study show that PWE ask various questions to pharmacists and need support not only about epilepsy, but also about life issues. In order to optimize healthcare resources, community pharmacists may take more roles in epilepsy management. Further involvement of pharmacists in epilepsy management may help create strong bonds between pharmacists and patients and overcome the barriers to getting misleading and insufficient information. Having complete and accurate information may enhance pharmacists' counseling of PWE. Pharmacists are open to having more training on epilepsy, and vocational training may be organized. Another issue is that there is a communication gap between pharmacists and physicians. Therefore, future studies can be performed to explore the underlying reasons for the lack of communication.

Ethics Committee Approval: This study was approved by the Izmir Katip Çelebi University Social Research Ethics Committee (No: 2020/09-04).

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Case Report

A clinical pharmacist's intervention on interaction between meropenem and valproic acid in paediatric inpatient clinic: A case report

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ABSTRACT

Background and Aims: Drug-drug interactions cause many complex drug events, especially adverse drug effects. It is known that drug interactions occur when meropenem and valproic acid are used together. It was aimed to back up existing research on the interaction of valproic acid.

Methods: After an interaction had been suspected by the ward pharmacists, all of the patient's drugs were assessed via UpToDate® and Micromedex® databases and the interaction management was shared with the physician in charge.

Results: The patient's serum valproic acid through level was in therapeutic range before meropenem administration. After the administration of meropenem, the patient's number of seizures increased and the serum trough level of valproic acid decreased to 4.3 µg/ml. The valproic acid levels turned back to normal range after switching to a carbapenem antibiotic.

Conclusion: Drug interactions are often encountered in paediatric patients and it is of critical importance in terms of seizure control. Because of the drug interaction between valproic acid and meropenem, one should avoid using these medications concomitantly. If that is impossible, daily serum valproic acid levels should be monitored with caution. Clinical pharmacists could increase the clinicians' awareness about drug-drug interactions.

Keywords: Drug interactions, hospitalized child, meropenem, clinical pharmacists, valproic acid

INTRODUCTION

Valproic acid (VPA) is widely used as an antiepileptic drug among paediatric patients (Miranda Herrero et al., 2015). The therapeutic range for serum VPA levels is 50–100 µg/ml for seizures (Wu, Pai, Hsiao, Shen, & Wu, 2016).

As being wide-spectrum antibiotics, carbapenems are used to treat infections caused by Gram positive, Gram negative aerob and anaerobic microorganisms (Wu et al., 2016).

It is reported in case reports and retrospective studies that carbapenems decrease serum VPA levels (Park et al., 2012; Spriet et al., 2007). The interaction between carbapenems and VPA was reported firstly in Japanese literature in the late 1990s (Al-Quteimat & Laila, 2020). In animal studies, it is shown that carbapenems cause the formation of valproate glucuronide and a rise in uridine diphosphate levels (Yamamura, Imura-Miyoshi, & Naganuma, 2000). Also, carbapenems inhibit the activity of VPA-glucuronidase

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in human liver cytosol (Nakamura, Nakahira, & Mizutani, 2008). Through these mechanisms, carbapenems can decrease VPA levels. The reduction in VPA levels rapidly happens in the first 24 hours of concomitant administration (Al-Quteimat & Laila, 2020). It is recommended to avoid the concomitant use of these two drugs (Spriet et al., 2007).

Here, we present a case regarding how the interaction happened and how clinical pharmacists can cope with that.

CASE PRESENTATION

A twelve-year-old female patient was admitted to an emergency department of a tertiary care hospital with complaints of fever, coughing, and breathing problems. Then, the patient was admitted to the general paediatric ward with pneumonia. The patient has cerebral palsy, epilepsy, and a gastrostomy feeding tube. The patient was receiving sodium valproate 400 mg q12h, zonisamide 100 mg q12h, ethosuximide 250 mg q8h and clobazam 15 mg q12h as epilepsy treatment via a gastrostomy feeding tube at home. The patient was experiencing seizure attacks up to four times daily which were lasting for about 20 seconds before admission.

On the 16th day of hospitalization, the patient's serum VPA through level (C0) was 62.5 µg/ml. On the 17th day, a sputum culture test was done and resulted in *Pseudomonas aeruginosa* and on the 23rd day of hospitalization, intravenous 1-gram meropenem q8h therapy was started.

On the 29th day, clinical pharmacists had suspected that a drug interaction might occur between meropenem and VPA, and questioned the patient's caregiver about the seizure status. Upon learning that the patient's number of seizure count had increased to twenty-five per day, the clinical pharmacists assessed all the patient's drugs via the UpToDate® and Micromedex® databases. The clinical pharmacists recommended measuring the serum VPA C0 to the physician in charge. On the 30th day, it was seen that the blood C0 of VPA had decreased to 4.3 µg/ml. Although the VPA had been transitioned from oral to intravenous form on the same day, the patient's number of seizure count had not decreased on the first day of intravenous VPA therapy. The patient's meropenem therapy had switched to intravenous piperacillin-tazobactam 320 mg q8h on the patient's 31st day of hospitalization.

On the patient's 32nd and 38th day of hospitalization, the serum VPA C0 increased to 19.2 µg/ml and 40.9 µg/ml, respectively. Alongside an increase in VPA trough concentrations, the patient's seizure frequency had decreased and on the 48th day, the patient was discharged. The interaction was assessed according to Naranjo Adverse Drug Reaction Probability Scale (Naranjo et al., 1981). The interaction could be classified as "probable" as it gets 7 scores according to the Naranjo Scale.

DISCUSSION

In a study, a 21-year-old female patient was admitted to an emergency department with seizure. Then, VPA 1000 mg over 24-hour infusion therapy was started. On the 13th day of hospitalization, an intravenous meropenem 1-gram q8h therapy was started. On the 15th day, the patient experienced seizure episodes. Despite increasing the VPA dose to 3600 mg, the serum level of VPA remained smaller than 10 µg/ml. On the 19th day, an interaction was suspected between meropenem and VPA and upon that, meropenem therapy was suspended. On the 27th day, the patient's level of VPA was 52.4 µg/ml. The Naranjo Probability Scale indicated that there could be a possible relationship between meropenem-VPA interaction and acute seizures (Coves-Orts, Borras-Blasco, Navarro-Ruiz, Murcia-Lopez, & Palacios-Ortega, 2005).

In another study, a 3-year-old boy was admitted to hospital and on the first day of hospitalization, meropenem therapy was started to treat pneumonia. On his 3rd day, because of his uncontrolled seizures and low serum VPA levels, his VPA dose was gradually increased. When meropenem therapy was switched, the VPA level reached 18.6 µg/ml (Okumura, Andreolio, Di Giorgio, Carvalho, & Piva, 2017).

In another study, a 5-year-old female patient was admitted to a hospital with severe respiratory failure. On her 10th day of hospitalization, meropenem therapy was started because of hemodynamic deterioration. Five days after the meropenem therapy was started, the serum VPA level was found to be smaller than 3 µg/ml and due to that, the meropenem therapy was suspended. After the dose of VPA was increased, the VPA concentration was found to be 55 µg/ml. Eventually, the patient died because of refractory hypoxemic failure (González & Villena, 2012).

Although it is recommended that if using these two drugs concomitantly is inevitable, another drug that belongs to a different antiepileptic drug class should be added to the patient's drug regimen temporarily; that is however, not always the case. In this case report, the concomitant use of more than one drug belonging to different antiepileptic classes was of no benefit because of the patient's resistant seizures.

In our study, it has been shown that converting the oral form of VPA to the intravenous form has failed to prevent this interaction to happen. However, our study has some limitations: the daily serum VPA levels were not determined, so it is unclear when the interaction started or whether the VPA levels reached the therapeutic range after the antibiotic treatment was switched.

CONCLUSION

In conclusion, drug interactions are often encountered in paediatric patients and it is of critical importance in terms of seizure control. Because of the drug interaction between VPA and meropenem, one should avoid using these medications concomitantly. If that is impossible, daily serum VPA levels should be monitored with caution.

Examining the literature, it is found that there is not as much research in Türkiye compared to studies around the world on clinical pharmacists' detection and the management of drug interactions in paediatric clinics.

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Review

The fine line between occupational exposure and addiction to propofol

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ABSTRACT

Addiction is considered as an occupational hazard, especially in anesthesia units in hospitals. Professionals in anesthesiology clinics have easy access to a wide range of potent psychoactive drugs, causing them to have a higher risk of drug abuse compared to other healthcare personnel. Recent studies and case reports have shown that abuse of anesthetic drugs used in these units has become a problem among health professionals, and awareness of the problem has increased. Propofol is widely used in anesthesia as a sedative-hypnotic prescription drug and in the literature, propofol abuse is mostly referred to in connection with medical and paramedical personnel. In this manuscript, we reviewed the pharmaco-kinetics, -dynamics effect of propofol, discussed toxicological outcomes of propofol abuse and possible institutional measures to be taken against addiction.

Keywords: Propofol, addiction, abuse, anesthetics

INTRODUCTION

Propofol (2,6-diisopropylphenol) is an intravenous anesthetic agent used for induction of moderate-to-deep sedation prior to surgical operations and distressing medical interventions (Klausz, Rona, Kristóf, Töro, 2009; O'Malley, 2010; Bryson & Frost, 2011). The chemical formula of propofol is $C_{12}H_{18}O$ and its structure is given in Figure 1 (Feng, Kaye, Kaye, 2017). In clinical settings, propofol is safe for providing procedural sedation and it has been used to induce and maintain general anesthesia since 1985 (Kirby, Colaw, & Douglas, 2009; Levy, 2011). In intensive care units (ICU), some advantages like rapid onset, short action time and rapid recovery make propofol an excellent agent for anesthesia and sedation (O'Malley, 2010). Because of its high lipophilicity, propofol has a rapid onset of action and a short-term narcotic effect (Klausz et al., 2009; Levy, 2011). Its minimal residual effects on the central nervous system (CNS) and the rapid return of the patient's consciousness are further advantages of propofol, and with this effect, it is ideal for use in plastic surgery (McCarver & Spear, 2010). Besides induction and/or maintenance of anesthesia, propofol is also used in clinics for procedural sedation and in ICU for sedation in intubated and mechanically ventilated patients. Furthermore, propofol has off-label uses for management of refractory status epilepticus in children and adults and for the treatment of refractory postoperative nausea and vomiting (Folino, Muco, Safadi, & Parks, 2022; Drugbank Online, 2022). In addition to its anesthetic effects, propofol is also used in cases of resistant seizures, resistant migraine and tension headaches, severe alcohol withdrawal and delirium tremors, and to facilitate rapid opiate detoxification (Bonnet, Harkener, Scherbaum, 2008; Sarff & Gold, 2010; Levy, 2011). However, though propofol is not currently classified as a controlled substance, numerous case reports and

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Figure 1. The chemical structure of propofol.

case studies present the abuse potential and addiction risks of propofol (Welliver, Bertrand, Garza & Baker, 2012 ; Han, Jung, Baeck, Lee & Chung, 2013; Gwiazda et al., 2021).

Substance use disorders are not only a major social problem but also a part of a global public health crisis and a serious health concern (Onaolapo et al., 2022). Substance abuse is defined in the American Psychological Association (APA) dictionary (APA, 2022a) as "a pattern of compulsive substance use marked by recurrent significant social, occupational, legal, or interpersonal adverse consequences, such as repeated absenteeism from work or school, arrests, and marital difficulties". According to the Diagnostic and Statistical Manual of Mental Disorders - Fifth Edition (DSM-V), substance abuse and dependence is defined as "substance use disorder" and diagnosing the disorders has been revised (APA, 2022a; Dugosh, 2022). DSM–V–TR places alcohol-, caffeine-, cannabis-, hallucinogens-, inhalants-, opioids-, sedatives-, hypnotics-, anxiolytics-, stimulants-, and tobacco-related disorders under substance-related and addictive disorders, and propofol addiction falls under the category of "sedative-, hypnotic-, or anxiolytic-related disorders." (APA, 2022b). According to Kirby et al., certain criteria can be used in the diagnosis of substance use disorders. These are: i. Compulsion or craving to use a substance; ii. loss of control over the amount or frequency of the substance used; iii. continued use of the drug despite adverse consequences and ensuing problems in relationships; and iv. neglecting normal life necessities (Kirby et al., 2009).

In this manuscript, after summarizing the pharmacological properties (pharmacokinetics, pharmacodynamics, adverse effects) of propofol, the toxicological outcomes including addiction are discussed.

Pharmacokinetic profile of propofol

A three-compartment linear model has been defined for propofol pharmacokinetics: i. a large central compartment ii. peripheral compartment with lower perfusion (lean tissues) iii. deep compartment with low perfusion (fatty tissues) (Iwersen-Bergmann, Rösner, Kühnau, Junge, Schmoldt, 2001; Kranioti, Mavroforou, Mylonakis, Michalodimitrakis, 2007; Klausz et al., 2009; Levy, 2011; Budic et al., 2022). For induction of anesthesia, 1.5-2.5 mg per body weight as kg dose of propofol is sufficient to produce unconsciousness. Children need greater doses while the elderly need smaller doses because of the pharmacodynamic variances (e.g., central distribution volumes and clearance rates) (McCarver & Spear, 2010). There is a sharp decrease in brain concentrations due to the redistribution from the central compartment into peripheral compartments, and this may result the patient waking up from anesthesia (Iwersen-Bergmann et al., 2001).

Propofol has a bitter taste and low oral bioavailability (high first pass effect and high hepatic extraction rate). Therefore, it can only be used intravenously. After intravenous administration, propofol is significantly bound to plasma proteins (mostly albumin) with 97-99% rate and erythrocytes (Budic et al., 2022). Propofol is metabolized very rapidly, and it leaves the tissue storage areas. Thus, plasma concentrations never increase (McCarver & Spear, 2010; Levy, 2011). Propofol is biotransformed to inactivate metabolites with UDP-glucuronosyltransferase (70%) and CYP2B6 and CYP2C9 (29%) enzymes to form either glucuronide or sulfate conjugates in the liver and then excreted by the kidney (1%) (Figure 2) (Iwersen-Bergmann et al. 2001; Kranioti et al., 2007; Klausz et al., 2009; McCarver & Spear, 2010; Levy, 2011; Dinis-Oliveira, 2018; Budic et al., 2022). Polymorphisms in these enzymes are responsible for the individual variables of propofol anesthetic effect like the unpredictable effects of standard doses or prolonged recovery time from anesthesia. The small intestines are also active in the metabolism of propofol with an 24% extraction ratio (Budic et al., 2022). Less than 1% is excreted unchanged in the urine and 2% in the feces. Since kidney and liver diseases do not affect propofol's excretion, it does not accumulate even in patients with those diseases (Levy, 2011). The elimination of propofol does not cause cirrhosis or renal failure, and its elimination half-life is between 0.5 and 1.5 hours (McCarver & Spear, 2010; Levy, 2011). Because of its high lipophilicity, propofol easily crosses the placental barrier and may cause neonatal CNS and respiratory depression (McCarver & Spear, 2010; Dugosh &Cacciola, 2022). If the neonates are exposed to propofol, they should be monitored for hypotonia and sedation (Dugosh &Cacciola, 2022). Animal studies have shown that blocking NMDA receptors and/or potentiate gama-aminobutyric acid (GABA) activity with the use of general anesthetic and sedation medications may affect brain development. Fetal exposure to propofol requires benefit/risk analysis if the exposure duration is greater than 3 hours (Olutoye, Baker, Belfort & Olutoye, 2018; Dugosh &Cacciola, 2022).

Only a small amount of propofol may be excreted to the milk and it is not expected to be absorbed by the infant. Mothers can breastfeed after sufficient recovery from general anesthesia, thus discarding the milk is unnecessary (LactMed, 2021).

Pharmacodynamic profile of propofol

Propofol exerts its pharmacological effect by increasing the chloride ions current at the GABA type-A receptors located in different areas (the reticular activating system, the chemoreceptor trigger zone, the medullary and pontine ventilator centers) (Roussin, Montastruc, Lapeyre-Mestre, 2007; Klausz et al., 2009; McCarver & Spear 2010; Sarff & Gold, 2010; Bryson & Frost, 2011; Levy, 2011) and it activates GABA to produce hypnosis (Budic, 2022). It may also interfere with and inhibit the functions of the N-methyl-D-aspartate receptors (NMDA), α-amino-3-hydroxy-5-

CYP2B6
CYP2C9
29%
UGT
70%
2,6-diisopropyl-1,4-quinol
Propofol
(2,6-diisopropylphenol)
Propofol-glucuronide PG
(major metabolite)
NQO1
UGT
1-(2,6-diisopropyl-1,4-quinol)-glucuronide 1-QG
2,6-diisopropyl-1,4-quinone
UGT
SULT
4-(2,6-diisopropyl-1,4-quinol)-glucuronide 4-QG
4-(2,6-diisopropyl-1,4-quinol)-sulphate
4-QS

Figure 2. Metabolic pathway of propofol (Dinis-Oliveira, 2018).
SULT: sulfotransferase; UGT: UDP-glucuronosyltransferase; NQO1: diaphorase; CYP: cytochrome P450.

methyl-4-isoxazolepropionic acid (AMPA) receptors, and nicotinic acetylcholine receptors. Moreover it activates inhibitory glycine receptors at the spinal cord level (Roussin et al., 2007; Sarff & Gold, 2010; Bryson & Frost, 2011; Levy, 2011; Feng et al., 2007; Budic et al., 2022). Propofol causes dissociation of GABA from receptors slowly and an increased duration of GABA-activated opening of chloride channels. The opening of chloride channels leads to hyperpolarization of cell membranes which in turn leads to non-response to external stimuli (McCarver & Spear, 2010; Budic et al., 2022). It also has effects on cerebral oxygen requirements, cerebral blood flow and intracranial pressures. Other pharmacological effects include anticonvulsant, antioxidant, anti-inflammatory and bronchodilator effects (Kranioti et al., 2007; McCarver & Spear, 2010).

It is reported that propofol has significant amnesia effects and that it reduces postoperative nausea and vomiting in plastic surgery. Thus, small doses of propofol may be effectively used in post-anesthesia care to treat vomiting and nausea. These small doses do not produce sedation and their anti-emetic action may exert a depressing effect on CNS structures including subcortical areas. Vomiting may also be directly depressed by propofol (McCarver & Spear, 2010).

Adverse effects of propofol

Propofol must be administered by experienced and qualified clinicians (Levy, 2011). However, some complications (e.g., bacteremia, sepsis, hypertriglyceridemia, pancreatitis and a propofol-infusion syndrome) have been found to be associated with its use and these complications are both severe and life-threatening (Sarff & Gold, 2010; Levy, 2011). However, asystole seems to occur only rarely with the administration of propofol. In the elderly, hypovolemia further enhances the hypotensive effects of propofol. Propofol causes a dose-dependent ventilator depression with apnea in 25-35% of inductions of anesthesia. Larger propofol doses can cause respiratory depression and even apnea, also benzodiazepines have sufficient cumulative effects resulting in apnea (McCarver & Spear, 2010). Respiratory and cardiovascular depression are major adverse reactions which have been observed in propofol use. It is clear that the use of propofol requires medical assistance (Roussin et al., 2007). Propofol may become potentially harmful and lethal when injected by incompetent personnel or self-administered because of its potent pharmacodynamics effects on the cardiovascular and respiratory systems (Levy, 2011). Increased rapidity of injection can lead to respiratory depression, and without ventilatory assistance this can lead to death (Roussin et al., 2007).

Because its lipid emulsion formulation can lead to growth of microorganism including *Escherichia coli*, Pseudomonas, *Staphylococcus aureus*, *Candida albicans,* it is important to pay attention to aseptic use when opening propofol vials and to apply it as soon as possible (McCarver & Spear, 2010; Levy, 2011).

Another potential side effect of propofol is hypertriglyceridemia (Roussin et al., 2007; Levy, 2011). When lipid emulsion is used (containing 0.1 g/ml fat infused for >72 h in ICU it can cause hyperlipidemia. Pancreatitis is the result of an increased dose in serum triglycerides (Levy, 2011). Propofol-infusion syndrome is another adverse effect characterized by severe metabolic acidosis, rhabdomyolysis, dyslipidemias, acute renal failure, hypotension, myocardial failure, bradyarrhythmia, and cardiac arrest. Risk factors associated with propofol include situations when propofol infusion (more than 4-5 mg/kg per hour for > 48 h or 67-83 mcg/kg/minute) is given with concomitant use of high dose steroids and catecholamine vasopressors. Other risk factors are cumulative doses of propofol, high fat low carbonhydrate intake, young age (<18 years), critical illness (airway infection, severe head trauma, sepsis, etc.) and inborn errors of mitochondrial fatty acid oxidation. People at particular risk are those on a ketogenic diet or those with carnitine deficiency (Levy, 2011; Diedrich & Brown, 2017; Fong et al., 2008; Mirrakhimov, Voore, Halytskyy, Khan & Ali, 2015). In addition, severe neurological damage, which may cause stress with increased catecholamines and glucocorticoids in circulation, may also be trigger factors in the development of propofol infusion syndrome (Hemphill, McMenamin, Bellamy & Hopkins, 2019)

Propofol abuse

Abuse of propofol is increasing due to its rapid onset of action (<1 minute) and short duration of action (5-10 minutes) (Kirby et al., 2009). Propofol has not typically been listed as a controlled substance (Kirby et al., 2009; McCarver & Spear, 2010; O'Malley, 2010; Levy, 2011) and it is used in operating rooms and ICU. (McCarver & Spear, 2010). Only a small dose required for pleasure, easy access, short duration for action, and without long term side effects make it preferable for those who abuse propofol (O'Malley, 2010; Kirby et al., 2009). Welliver et al. (2012) reported two cases of propofol abuse. The first case was an anesthesiology nurse without a history of substance abuse. After an abdominal hysterectomy, the nurse was given an infusion of fentanyl 4-5 times of the normal dose as a result of a prescription error. The nurse self-administered intravenous (iv) fentanyl within 2 weeks of returning to work. At the end of 7 months, she started to use propofol due to the difficulty in obtaining increased fentanyl doses and in order to reduce the withdrawal syndrome caused. Propofol caused both a decrease in leg shaking, which is a symptom of fentanyl withdrawal syndrome, and euphoria. The nurse continued to use propofol, gradually increasing the dose to experience euphoria, and had a serious car accident after using the substance. After she was found unconscious in the vehicle, she was treated at the rehabilitation center on suspicion of drug abuse. In the second case, the propofol abuser was also an anesthesiology nurse. She used ketorolac after a painful ankle injury but switched to opioids due to the need for more pain relief. She used fentanyl and morphine in increasing doses for 4-6 months, and when it became difficult to obtain these agents, she started using propofol due to its easy access. She continued to use it in the hospital, and her unsteady gait, incidents of falling, and black eyes made her colleagues suspect something which caused them to intervene. The nurse accepted that she had a problem and was directed to therapy. But just after her treatment, she went back to one of the operating rooms in her workplace and used propofol again. After this she received treatment voluntarily. In both cases, each person preferred propofol because of its ease of access. In addition, they could not stop using propofol despite personal injuries and occupational impairments (Welliver et al., 2012)

Two hypotheses arise in the field. The first assumes that patients do not generally know which drug is used for the induction of anesthesia and in most patients who are exposed to propofol, dependency is not a matter of concern. The other hypothesis focusses on some underlying psychiatric pathology or a previous history of drug addiction (Fritz & Niemczyk, 2002). In many cases, the patient history includes drug dependency (Levy, 2011). Propofol addicts cannot cope with the pharmacological effects of the drug, and they may fall and injure themselves after administration (McCarver & Spear, 2010).

A survey made by Wischmeyer et al. searched the information about individuals abusing propofol over the last decade in the USA. They reported that 18% of the hospital departments had one or more people abusing propofol, and two of these departments had more than one incident. There were seven reported deaths linked to propofol abuse, and in each case evidence o abuse could only be discovered when the person was found dead (Wischmeyer et al., 2007). The study results also highlighted an age-related increase in the incidence of suicide in anesthesiology, and a decrease in drug-related deaths due to age. Propofol's short half-life and narrow safety window make its abuse difficult to detect (Wischmeyer et al., 2007). A systematic review on suicide in anesthetics stated that the proportion of anesthetists dying by suicide had increased, and it was emphasized that anesthetic drugs, especially propofol had been used. The study was not specifically conducted to investigate substance abuse or addiction among anesthetists, however propofol was notably seen to be a suicidal drug of abuse among anesthetists. An internet search of deaths due to propofol abuse found that 18 (86%) of 21 fatal cases among healthcare workers were in anesthesia units most of whom were medical or nurse anesthetists (Plunkett, Costello, & Yentis, 2021)

A case from Turkiye was reported in 2015. An emergency medicine doctor who used propofol help relieve his pain caused by nephrolithiasis, was admitted to the psychiatry clinic because of his propofol addiction. After two weeks he refused therapy and left the clinic. Two years later he was found dead due to suspected propofol overdose in the hospital where he had worked (Köroğlu & Tezcan, 2015). In a study by Han et al. the prevalence of propofol abuse was researched using data on blood concentrations of all autopsy cases performed at Korea's National Forensic Service in 2005-2010. Of the 14,673 autopsy cases, propofol was detected in 131, and among these, it was documented that 16 deaths had occurred because of propofol abuse. Also, it was found that nurses and physicians were involved in half of the cases. Moreover, the same study reported that propofol was used more frequently by female healthcare professionals within the 20-30 age range (Han et al.,2013). The results of a retrospective study conducted in Australia and New Zealand by Fry et al.

in 2004-2013, showed that substance abuse was a major problem and associated with a high mortality rate. The mortality rate associated with abuse of propofol was 45% (Fry, Fry & Castanelli, 2015). Furthermore, Gwiazda et al. reviewed two recent case reports for awareness of propofol abuse in Ireland. The first case was a woman found dead at the institution where she worked as a healthcare worker. During the autopsy, it was found that she had old needle puncture marks and an intravenous cannula. Propofol was found in the intravenous infusion bag. However, propofol had not been detected in the post mortem femoral blood and urine samples. This may be explained through the short-acting profile of propofol and inter-individual pharmacokinetic variability. The second case was of a healthcare worker who was found dead in his bed. Used propofol vials were noted in the scene investigation. In the toxicological analysis, both propofol and its glucuronide conjugate were detected in the femoral blood sample, of note with a lethal concentration of propofol (Gwiazda et al., 2021).

A number of factors including access to these addictive drugs and the relative ease of reserving them in small amounts for personal use, as well as high-stress at work, and occupational exposure-related sensitization and stimulated reward pathways in the brain may explain the higher incidence of drug use among anesthetists (Bryson, Silverstein, Warner & Warner, 2008; Merlo, Goldberger, Kolodner, Fitzgerald & Gold, 2008). The largest cohort of known propofol abusers is represented by medical professionals and healthcare workers (Levy, 2011). Propofol abuse by these people has increased, especially among anesthesiology professionals who are younger than 35 years of age and have easy access to the drug (Roussin et al., 2007; Li, Xiao, Xiong, Delphin & Ye, 2008; Merlo et al., 2008; McCarver & Spear, 2010; O'Malley, 2010; Levy, 2011) According to Mensch and Kandel, certain personality characteristics like being highly achievement-oriented, self-controlled, independent, and less comfortable asking for help from others are common for anesthesiologists, and this may contribute to their increased risk of addiction (Mensch & Kandel 1988). A literature review on fatal cases involving propofol abuse revealed that 86% of the cases were healthcare professionals, especially anesthesiologists and nurse anesthetists. Moreover, most of these cases were documented as accidental (81%), with only 9.5% being represented by homicide, and only 9.5% by suicide (Diaz & Kaye, 2017). In another retrospective case study conducted by Earley et al. medical records of healthcare professionals treated at an addiction center were examined. According to the records, 50% of the healthcare personnel had used propofol and most of the propofol abusers worked in the operating room, being women and anesthesiologists. Of note, people who were dependent on propofol often had a history of depression (Earley & Finver, 2013). Propofol, which has the potential for abuse, has taken its place among controlled substances in South Korea since 2011. In this context, Cho et al. conducted a study, using the South Korean Supreme Court database to analyze the criminal cases related to the abuse of propofol by healthcare professionals between 2013-2020. In this study, criminal cases related to the abuse of propofol by healthcare professionals were analyzed. Finally, it was concluded that propofol abuse in this study was the most common among nurses (Cho, Hwang, Shin, Yoon & Lee, 2022).

Psychological addiction is observed to be more common than physical addiction due to various factors such as associated euphoria, relief from stress and tension, post-injection, and post-waking sexual fantasies and dreams. These effects lead to drug craving and loss of control over the amount and frequency of propofol injection which defines psychological dependence (Levy, 2011). It is assumed that psychological dependence might be a result of the rapid activation of mesolimbic GABA-A receptors (Bonnet et al., 2008). Moreover, repeated injections exceeding one hundred per day were reported in chronic propofol abusers (Levy, 2011). Xylocaine significantly reduces pain when administered intravenously but does not alter the effects of propofol (Kranioti et al., 2007).

Like most drugs of abuse, propofol also increases dopamine levels by directly blocking dopamine release or dopamine reuptake from presynaptic nerve terminals (Roussin et al., 2007; Bryson & Frost, 2011; Levy, 2011). This effect may be the underlying factor for the abuse potential of propofol. Propofol is known to cause visual hallucinations by inhibiting NMDA receptors in the brain, similar to ketamine, another abused anesthetic (Levy, 2011). Reported effects range from feeling intense pleasure, vivid dreams related to sex, relaxation, disinhibition and euphoria to unconsciousness and apnea (Roussin et al., 2007; Klausz et al., 2009; McCarver & Spear, 2010; O'Malley, 2010; Levy, 2011). There are some reported reasons which typically lead to the self-administration of propofol for recreation, stress reduction or preventing insomnia (Roussin et al., 2007; O'Malley, 2010).

Occupational exposure to propofol used during surgery may sensitize anesthetists and surgeons to its effects and may subsequently lead to it being abused. (Wischmeyer et al., 2007). Gold et al. suggested that anesthetists who become addicted because of occupational exposure may continue to use agents to mitigate drug withdrawal effects when away from exposure (Gold et al., 2006). In this regard, operating rooms may be regarded as a toxic working environment for anesthesiologists. Because some individuals are more vulnerable to second-hand exposure than others, many anesthetists may become addicted due to susceptibility (Merlo et al., 2008). In addition, recent studies suggest that occupation-related second-hand exposure to intravenous drugs, including propofol (McAuliffe et al., 2006; Levy, 2011), may occur. McAuliffe and colleagues tested this hypothesis and showed that second-hand exposure may increase the risk of substance addiction. Occupational exposure to anesthetics and opiates due to increased indoor air concentration in the workplace sensitizes the brain and this may increase the risk of addiction. In the operating room, the levels of propofol or fentanyl cannot be readily detectable; however, it was suggested by McAuliffe et al. that intravenously injected fentanyl or propofol can be found in the operating room environment but may only be detected using highly sensitive techniques. Such an occupational exposure can lead to addiction and, in the end, people who work in operating rooms may experience withdrawal symptoms (McAuliffe et al., 2006). These possible outcomes were also confirmed by Merlo et al. (Merlo et al., 2008).

There are symptoms which indicate substance dependence such as reducing or completely stopping indispensable activities, intaking an increased amount of the substance, spending excessive time to obtain it, inability to control its use, using the substance or recovering from its use, drug tolerance, withdrawal symptoms, and continuing its use despite adverse consequences. If three or more of the above symptoms occur within one-year period substance dependence is defined (McAuliffe et al., 2006). Patients who use propofol against symptoms such as chronic headaches or migraines may develop psychological dependence. This is an underlying psychological imbalance or disorder with or without dependence on other drugs. Most abusers do not develop true dependence on propofol because there is no evidence of tolerance (Kranioti et al., 2007). However, tolerance may also occur if the frequency of injections is significantly increased (> 100 times a day) (Iwersen-Bergmann et al., 2001).

There are no physical dependence characteristics to define propofol addiction (Roussin et al., 2007). However, symptoms of addiction in healthcare professionals are summarized as follows (i) unusual behavior changes, (ii) mood changes including euphoria, and depression; (iii) desire to work alone and without taking a break; (iv) volunteering for extra cases and withdrawing from social environments with family and friends, avoiding social activities; (v) increase in anger and irritability; (vi) going to work early and/or leaving work late even when off duty; (vii) physical changes like weight loss and pale skin, frequent desire to take breaks while working; (viii) requesting inappropriate and/or increasing amounts of abused substance (Kirby et al., 2009; O'Malley, 2010; Bryson & Frost, 2011). Also, the half-life of the abused drug may affect the tolerance rate (Bryson & Silverstein, 2008).

Measures taken for propofol

Addiction is a significant problem among health professionals with serious implications for public health (McAuliffe et al., 2006; Merlo et al., 2008). Increase in suicide and general mortality rates among physicians with substance abuse may affect their job performance and can harm their patients (Merlo et al., 2008). Monitoring the level of the possible exposure in the biological materials of healthcare professionals may be a way to take some precautions against second-hand occupational exposure. For this purpose, a variety of analytical methods have been developed for the detection and quantification of propofol in different sample types obtained from abusers. High-performance liquid chromatography and gas chromatography are widely used techniques for detection (Levy, 2011). In order to demonstrate chronic abuse, hair analysis of propofol has become an accurate and preferred method (O'Malley, 2010). Also, Kwon et al. (2020) developed and validated a simple, fast, and sensitive LC-MS/MS method for the determination of propofol-glucuronide in hair samples to identify chronic use of propofol. The study also sought to investigate the relationship between dose and hair concentration. While a positive relationship has been observed between dose and hair concentration in some subjects, others with recent hair treatment presented uncorrelated results which has been attributed to variations between individuals like irregular hair growth, hair treatment or contribution from sebum (Kwon, Kim, Cho, Lee & Han, 2020)

Anesthesia locations, ICUs, hospital pharmacies and emergency departments are potential places where drug abuse is common in hospitals. In recent years, many pharmacies in hospitals and anesthesia units have created systems to prevent diversion since propofol abuse is being recognized around the world. Furthermore, some hospitals have installed systems that allow pharmacies to use a fingerprint identification system to track access. These systems keep track of the number of drugs in the pharmacy and frequency of use. Moreover, some healthcare institutions keep pharmacy and anesthesia records in order to detect inconsistencies. Others consider that propofol should be a controlled substance and recommend routine testing of propofol in suspected or at-risk individuals with potential for addiction (Levy, 2011). Treatment for addiction is multifaceted and includes detoxification, follow-up abstinence, as well as education and psychotherapy. During this period, patients are kept away from stress and from access to medication (Bryson & Silverstein, 2008).

CONCLUSION

Propofol is a short acting anesthetic agent used intravenously for sedation against painful or uncomfortable procedures. Propofol abuse has been increasing in recent years for various reasons such as its property of rapid onset, the small dose required for pleasurable effects, the short duration of action, and ease of access. However, when propofol is used for purposes of abuse, it can cause psychological dependence, and this can lead to mortality. Generally, propofol is a drug used in operating/anesthesia rooms and ICUs and is not listed as a controlled substance. Furthermore, several published articles and case reports have shown the problems experienced by propofol abusers. Studies and various case reports have proved that propofol abuse is prevalent among healthcare specialists. In this context, it is mainly anesthetists and nurse anesthetists who are at risk. Considering the increase in propofol abuse by healthcare professionals and the potential risk for job interruptions, increased morbidity and mortality, accidents, and deterioration of public health caused by this abuse, new regulations should be made for the safe and controlled use of propofol.

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