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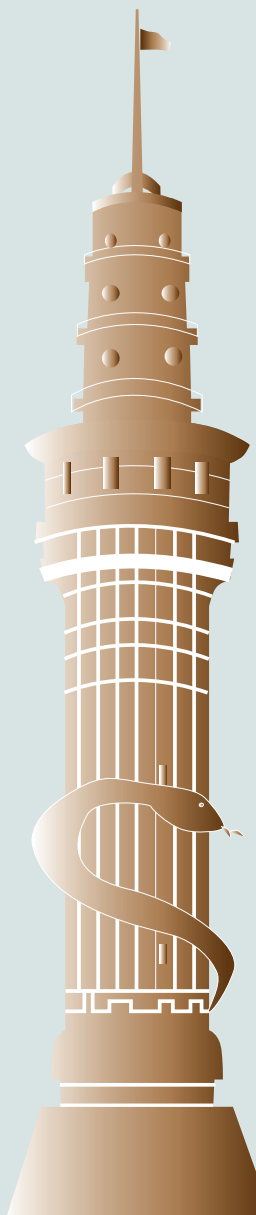

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İstanbul Journal of Pharmacy (Istanbul J Pharm) aims to contribute to the literature by publishing manuscripts at the highest scientific level on all fields of pharmaceutical sciences. The journal publishes original articles, short reports, letters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

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4. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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- Acknowledgment of the individuals who contributed to the preparation of the manuscript but who do not fulfill the authorship criteria.

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Short Paper	1000	200	No tables	10 or total of 20 images
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When a drug, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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REFERENCES

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- American Psychological Association. (2010). Publication manual of the American Psychological Association (6th ed.). Washington, DC: APA.
- http://www.apastyle.org

Accuracy of citation is the author's responsibility. All references should be cited in text. Reference list must be in alphabetical order. Type references in the style shown below

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Citations must be indicated with the author surname and publication year within the parenthesis.

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More than one citation;

(Esin et al., 2002; Karasar, 1995)

Citation with one author;

(Akyolcu, 2007)

Citation with two authors;

(Sayıner & Demirci, 2007)

Citation with three, four, five authors;

First citation in the text: (Ailen, Ciambrune, & Welch, 2000) Subsequent citations in the text: (Ailen et al., 2000)

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Citations in the Reference

All the citations done in the text should be listed in the References section in alphabetical order of author surname without numbering. Below given examples should be considered in citing the references.

Basic Reference Types

Book

a) Turkish Book

Karasar, N. (1995). *Araştırmalarda rapor hazırlama* (8th ed.) [Preparing research reports]. Ankara, Turkey: 3A Eğitim Danışmanlık Ltd.

b) Book Translated into Turkish

Mucchielli, A. (1991). *Zihniyetler* [Mindsets] (A. Kotil, Trans.). İstanbul, Turkey: İletişim Yayınları.

c) Edited Book

Ören, T., Üney, T., & Çölkesen, R. (Eds.). (2006). *Türkiye bilişim ansiklopedisi* [Turkish Encyclopedia of Informatics]. İstanbul, Turkey: Papatya Yayıncılık.

d) Turkish Book with Multiple Authors

Tonta, Y., Bitirim, Y., & Sever, H. (2002). T*ürkçe arama motorlarında performans değerlendirme* [Performance evaluation in Turkish search engines]. Ankara, Turkey: Total Bilişim.

e) Book in English

Kamien R., & Kamien A. (2014). *Music: An appreciation.* New York, NY: McGraw-Hill Education.

f) Chapter in an Edited Book

Bassett, C. (2006). Cultural studies and new media. In G. Hall & C. Birchall (Eds.), *New cultural studies: Adventures in theory* (pp. 220–237). Edinburgh, UK: Edinburgh University Press.

g) Chapter in an Edited Book in Turkish

Erkmen, T. (2012). Örgüt kültürü: Fonksiyonları, öğeleri, işletme yönetimi ve liderlikteki önemi [Organization culture: Its functions, elements and importance in leadership and business management]. In M. Zencirkıran (Ed.), *Örgüt sosyolojisi* [Organization sociology] (pp. 233–263). Bursa, Turkey: Dora Basım Yayın.

h) Book with the same organization as author and publisher

American Psychological Association. (2009). *Publication manual of the American psychological association* (6th ed.). Washington, DC: Author.

Article

a) Turkish Article

Mutlu, B., & Savaşer, S. (2007). Çocuğu ameliyat sonrası yoğun bakımda olan ebeveynlerde stres nedenleri ve azaltma girişimleri [Source and intervention reduction of stress for parents whose children are in intensive care unit after surgery]. *Istanbul University Florence Nightingale Journal of Nursing, 15*(60), 179–182.

b) English Article

de Cillia, R., Reisigl, M., & Wodak, R. (1999). The discursive construction of national identity. *Discourse and Society, 10*(2), 149–173. http://dx.doi.org/10.1177/0957926599010002002

c) Journal Article with DOI and More Than Seven Authors

Lal, H., Cunningham, A. L., Godeaux, O., Chlibek, R., Diez-Domingo, J., Hwang, S.-J. ... Heineman, T. C. (2015). Efficacy of an adjuvanted herpes zoster subunit vaccine in older adults. *New England Journal of Medicine, 372,* 2087–2096. http://dx.doi.org/10.1056/NEJMoa1501184

d) Journal Article from Web, without DOI

Sidani, S. (2003). Enhancing the evaluation of nursing care effectiveness. *Canadian Journal of Nursing Research, 35*(3), 26-38. Retrieved from http://cjnr.mcgill.ca

e) Journal Article wih DOI

Turner, S. J. (2010). Website statistics 2.0: Using Google Analytics to measure library website effectiveness. *Technical Services Quarterly, 27,* 261–278. http://dx.doi.org/10.1080/07317131003765910

f) Advance Online Publication

Smith, J. A. (2010). Citing advance online publication: A review. *Journal of Psychology.* Advance online publication. http://dx.doi.org/ 10.1037/a45d7867

g) Article in a Magazine

Henry, W. A., III. (1990, April 9). Making the grade in today's schools. *Time, 135,* 28–31.

Doctoral Dissertation, Master's Thesis, Presentation, Proceeding

a) Dissertation/Thesis from a Commercial Database

Van Brunt, D. (1997). *Networked consumer health information systems* (Doctoral dissertation). Available from ProQuest Dissertations and Theses database. (UMI No. 9943436)

b) Dissertation/Thesis from an Institutional Database

Yaylalı-Yıldız, B. (2014). *University campuses as places of potential publicness: Exploring the politicals, social and cultural practices in Ege University* (Doctoral dissertation). Retrieved from Retrieved from: http://library.iyte.edu.tr/tr/hizli-erisim/iyte-tez-portali

c) Dissertation/Thesis from Web

Tonta, Y. A. (1992). *An analysis of search failures in online library catalogs* (Doctoral dissertation, University of California, Berkeley). Retrieved from http://yunus.hacettepe.edu.tr/~tonta/yayinlar /phd/ickapak.html

d) Dissertation/Thesis abstracted in Dissertations Abstracts International

Appelbaum, L. G. (2005). Three studies of human information processing: Texture amplification, motion representation, and figure-ground segregation. *Dissertation Abstracts International: Section B. Sciences and Engineering, 65*(10), 5428.

e) Symposium Contribution

Krinsky-McHale, S. J., Zigman, W. B., & Silverman, W. (2012, August). Are neuropsychiatric symptoms markers of prodromal Alzheimer's disease in adults with Down syndrome? In W. B. Zigman (Chair), *Predictors of mild cognitive impairment, dementia, and mortality in adults with Down syndrome.* Symposium conducted at the meeting of the American Psychological Association, Orlando, FL.

f) Conference Paper Abstract Retrieved Online

Liu, S. (2005, May). *Defending against business crises with the help of intelligent agent based early warning solutions.* Paper presented at the Seventh

International Conference on Enterprise Information Systems, Miami, FL. Abstract retrieved from http://www.iceis.org/iceis2005/abstracts_2005.htm

g) Conference Paper - In Regularly Published Proceedings and Retrieved Online

Herculano-Houzel, S., Collins, C. E., Wong, P., Kaas, J. H., & Lent, R. (2008). The basic nonuniformity of the cerebral cortex. *Proceedings of the National Academy of Sciences, 105,* 12593–12598. http://dx.doi.org/10.1073/pnas.0805417105

h) Proceeding in Book Form

Parsons, O. A., Pryzwansky, W. B., Weinstein, D. J., & Wiens, A. N. (1995). Taxonomy for psychology. In J. N. Reich, H. Sands, & A. N. Wiens (Eds.), *Education and training beyond the doctoral degree: Proceedings of the American Psychological Association National Conference on Postdoctoral Education and Training in Psychology* (pp. 45–50). Washington, DC: American Psychological Association.

i) Paper Presentation

Nguyen, C. A. (2012, August). *Humor and deception in advertising: When laughter may not be the best medicine.* Paper presented at the meeting of the American Psychological Association, Orlando, FL.

Other Sources

a) Newspaper Article

Browne, R. (2010, March 21). This brainless patient is no dummy. *Sydney Morning Herald, 45.*

b) Newspaper Article with no Author

New drug appears to sharply cut risk of death from heart failure.(1993, July 15). *The Washington Post,* p. A12.

c) Web Page/Blog Post

Bordwell, D. (2013, June 18). David Koepp: Making the world movie-sized [Web log post]. Retrieved from http://www.davidbordwell.net/blog/page/27/

d) Online Encyclopedia/Dictionary

Ignition. (1989). In *Oxford English online dictionary* (2nd ed.). Retrieved from http://dictionary.oed.com

Marcoux, A. (2008). Business ethics. In E. N. Zalta (Ed.). *The Stanford encyclopedia of philosophy.* Retrieved from http://plato.stanford.edu/entries/ethics-business/

e) Podcast

Dunning, B. (Producer). (2011, January 12). *inFact: Conspiracy theories* [Video podcast]. Retrieved from http://itunes.apple.com/

f) Single Episode in a Television Series

Egan, D. (Writer), & Alexander, J. (Director). (2005). Failure to communicate. [Television series episode]. In D. Shore (Executive producer), *House;* New York, NY: Fox Broadcasting.

g) Music

Fuchs, G. (2004). Light the menorah. On *Eight nights of Hanukkah* [CD]. Brick, NJ: Kid Kosher..

REVISIONS

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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İstanbul Journal of Pharmacy

CONTENTS

ORIGINAL ARTICLES

Istanbul J Pharm 50 (1): 1-5
DOI: 10.26650/IstanbulJPharm.2019.0038
Original Article

Determination of sultamicillin in bulk and pharmaceutical preparations via derivatization with NBD-Cl by spectrophotometry

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[2]University of Health Science, Pharmacy Faculty, Department of Analytical Chemistry, İstanbul, Turkey

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Cite this article as: Ozdemir, E., Taskin, D., & Sungur, S. (2020). Determination of sultamicillin in bulk and pharmaceutical preparations via derivatization with NBD-Cl by spectrophotometry. *İstanbul Journal of Pharmacy, 50*(1), 1-5.

ABSTRACT

Background and Aims: A new sensitive and selective method for the determination of sultamicillin in bulk and pharmaceutical preparations has been developed.
Methods: The developed method is based on the derivatization of sultamicillin with 4-chloro-7-nitrobenzofurazan in pH 9.0 borate buffer at 70°C for 60 min. The derivative was measured spectrophotometrically at 432 nm after extraction with chloroform.
Results: The method was validated by linearity, LOD, LOQ, accuracy, precision and recovery parameters. The linear range for sultamicillin was 10-50 $\mu g.mL^{-1}$ with a correlation coefficient of 0.9999. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 1.47 and 4.41 $\mu g/mL^{-1}$, respectively.
Conclusion: The suggested method was validated, and can be applied for routine sultamicillin analysis in dosage formsfor industrial analysis.

Keywords: 4-chloro-7-nitrobenzofurazan, sultamicillin, spectrophotometry, validation

INTRODUCTION

Sultamicillin is a mutual prodrug of ampicillin and sulbactam. Chemically, it is a double ester in which ampicillin and sulbactam are linked via a methylene group (Ph. Eur. 6th. edition). It is used for the treatment of childhood pneumonia and other pediatric infections, as oral and parenteral preparations (Ganesh, Kalshetti & Sanket, 2018). Sultamicillin tosylate dihydrate ($C_{32}H_{38}N_4O_{12}S_3.2H_2O$) is 4-Methylbenzenesulphonate of methylene (2S,5R,6R)-6-[[(2R)-aminophenylacetyl]amino]-3,3-dimethyl7-oxo-4-thia-1 azabicyclo[3.2.0]heptane-2-carboxylate (2S,5R)-3,3-dimethyl-4,4,7-trioxo-4λ^6 -thia-1- azabicyclo[3.2.0]heptane-2-carboxylate dihydrate (Ph. Eur. 6th. edition).

4-chloro-7-nitrobenzofurazan (NBD-Cl) is widely used as a chromogenic and fluorogenic reagent for the spectrophotometric and spectrofluorometric (Ayad et al, 2013; Azza et al., 2010; Darwish et al., 2008; El-Abd et al., 2013; El-Enany et al., 2009; Klimisch & Stadler, 1974; Olgun et al., 2002; Shehata et al., 2006; Taha et al., 2006), chromatographic (Azza et al., 2010; El-Emam et al., 2004; Farshchia et al., 2009; Murray et al., 1983; Tatar Ulu, 2007; Tosunoglu et al., 1995; Yılmaz et al., 2016) determinations of pharmaceutical amines.

One spectrophotometric (Kshirsagar et al., 2018) anda few liquid chromatographic methods (El-Shanawani, 1998; Pajchel & Tyski, 2002; Laviana, Fernandez, Bayod & Blanco, 2003; Ganesh et al., 2018) have been reported previously for the quantitative

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determination of sultamicillin in pharmaceutical dosage form. In the method of Kshirsagar et al. 2018 absorbance measurements were made at 232 nm which is the absorption maximum of sultamicillin in methanol. For the assay of sultamicillin in pharmaceutical dosage forms, more selective analytical methods are required to avoid possible interferences of the excipients. So, in the present study, developing a new spectrophotometric method is aimed, involving derivatization with NBD-Cl, chloroform extraction and absorbance measurement of the derivative.

MATERIALS AND METHODS

Reagents

Sultamicillin tosylate dihydrate was kindly supplied by Deva (Istanbul, Turkey). and NBD-Cl was purchased from Sigma Aldrich (St. Louis, MO, USA). The 375 mg sultamicillin tablets were purchased from a local pharmacy. Other chemicals were provided by Merck (Darmstadt, Germany). All solvents used were of analytical grade.

Apparatus

A Shimadzu UV-mini 1240 PC-UV visible spectrophotometer with 1 cm quartz cell was used for all spectral measurements. pH measurements were carried out with a Jenco 6179 pH meter.

Preparation of standard and working stock solution

The stock solution of sultamicillin was prepared in the concentration of 10 µg.mL^{-1} by dissolving the appropriate amount of sultamicillin tosylate dihydrate in methanol. A working standard solution of sultamicillin containing 2 mg.mL^{-1} was prepared by an appropriate dilution of the stock solution with methanol. A 2.0 mg.mL^{-1} NBD-Cl solution was prepared in methanol. The reagent solution was stable for two weeks if kept at ±4ºC (Taha et al., 2006). Borate buffers were prepared in the pH range of 8 to 10.

General analytical procedure

25-125 µL aliquots of solution containing sultamicillin tosylate dihydrate equivalent to 50-250 µg sultamicillin, were transferred into a series of reaction tubes. To each tube, 200 µL of pH 9 borate buffer and 150 µL of NBD-Cl solution (2 mg.mL^{-1}) were added and mixed in a vortex mixer. Then, these were kept at 70ºC for 60 minutes, cooled to room temperature and acidified with 100 µL of 0.1 M HCl. After extracting with 2x2 mL of chloroform, the extracts were combined and diluted to 5 mL with chloroform. The measure of absorbance was 432 nm against the blank solution.

Procedure for tablets

A portion of sultamicillin tablet powder obtained from 20 tablets, equivalent to 200 mg sultamicillin, was transferred into a 100 mL calibrated flask, sonicated with 50 mL of methanol for 30 minutes, completed to its volume with methanol, mixed well and filtered. The first portion of the filtrate was rejected. A 75 µL of aliquot of the solution was used for the determination.

RESULTS

NBD-chloride reacts with aliphatic primary and secondary amines to produce coloured and fluorescent derivatives. NBD derivatives have some advantages such as good stability, having an absorption maximum in the visible region and high signal/noise ratios (Bahrami & Mohammadi, 2006). Sultamicillin reacts with this reagent in alkaline buffer medium via its primary amino group yielding a yellow coloured product. Figure 1 shows the reaction between sultamicillin and NBD-Cl. The maximum absorbance of this derivative is at 432 nm in chloroform (Figure 2).

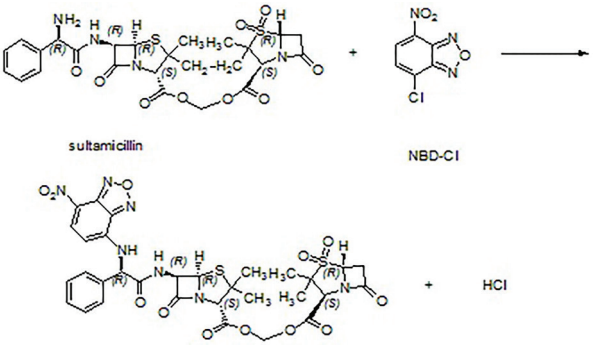

Figure 1. Reaction of sultamicillin with NBD-Cl.

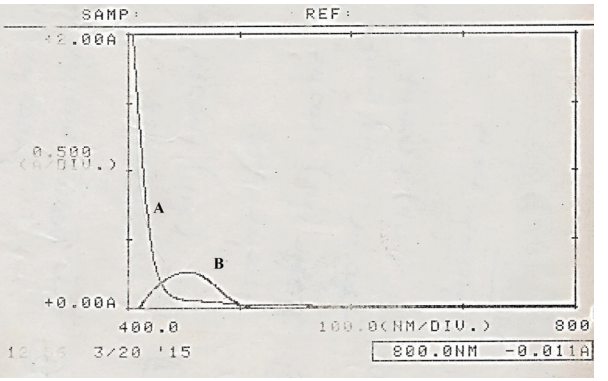

Figure 2. The absorption spectrum of sultamicillin-NBD derivative (B) and blank solution (A).

The conditions for the derivatization reaction were optimized. For this purpose, optimum pH, temperature, reaction time, amount of the reagent, extraction solvent and wavelenght were studied.

Effect of the temperature and reaction time: In order to determine the optimum temperature and reaction time for the derivatization reaction, room temperature, and 40, 50, 60 and 70°C temperatures were studied for 10-70 min intervals. The experimental results, shown in Figure 3, indicated that maximum absorbance was obtained in 60 min at 70°C.

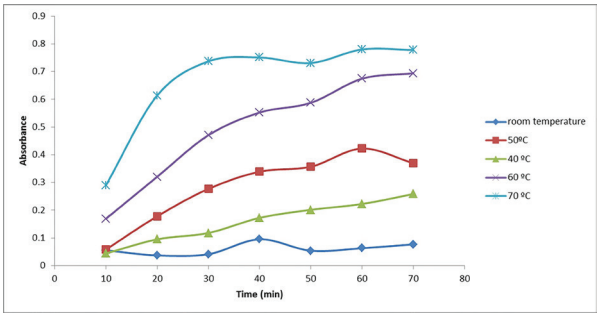

Figure 3. Effect of temperature and heating time on the derivatization (50 µg.mL^{-1} sultamicillin) .

Effects of pH: The reaction between sultamicillin and NBD-Cl was studied in the pH range of 8 to 10 intervals to determine the best pH at which the reaction was carried out, and the optimal pH was found to be 9.0. Figure 4a shows the effect of pH on the derivatization. As it is seen, max. absorbance was obtained in a pH 9 borate buffer. In the derivatization reaction, excess NBD-Cl is hydrolized to NBD-OH, which is extractable into chloroform and has an absorption at 432 nm. Therefore, the solution was acidified by adding 0.1 mL of 0.1 M HCl solution before the extraction step.

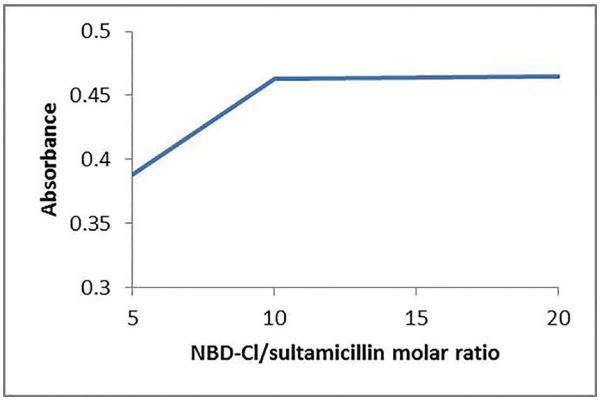

Figure 4a. Effect of reagent NBD concentration (30 µg.mL^{-1}).

Effects of reagent concentration: The amount of the reagent required for the maximum yield of the derivatization reaction, molar ratio of NBD-Cl/sultamicillin ranging from 5 to 20, was studied. As it is shown in Figure 4b, 10 fold molar excess of the reagent is sufficient to complete the reaction.

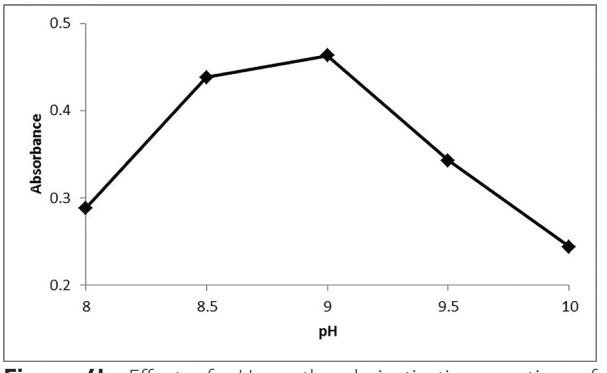

Figure 4b. Effect of pH on the derivatization reaction of sultamicillin (30 µg.mL^{-1}) with NBD-Cl.

Extraction solvent selection: Various organic solvents such as chloroform, ethyl acetate, methanol, dichloromethane, n-hexane were tested for the extraction of the derivative, and chloroform was chosen as the best solvent for the extraction, since the highest absorption intensity was observed in chloroform.

Method validation

The described method was validated as described in the International Conference on Harmonization (ICH) for drug applications (ICH Guideline, Q2(R1) 2005) in terms of linearity, LOD, LOQ, accuracy, precision and recovery.

*Linearity.*Under the optimum conditions, a linear relationship was obtained from five points covering the concentration range of 10-50 µg.mL^{-1}. The regression equation of the calibration curve was A=0.0157C - 0.004 (R^2=0.9999). The calibration curve is shown in Figure 5.

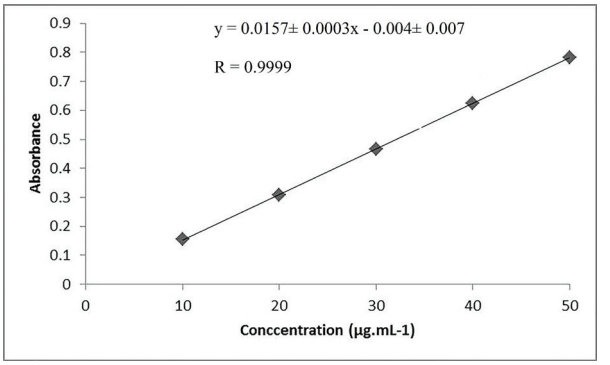

Figure 5. Calibration curve.

Limit of detection and limit of quantitation: The limit of dedection was calculated by LOD=3.3σ/S, where σ and S are the standart deviation of the intercept and the slope of the calibration curve. The limit of quantification was calculated as LOQ=10 σ/S. LOD and LOQ values were found to be 1.47 and 4.41 µg.mL^{-1} respectively. The statistical data are given in Table 1.

Table 1. Optical characteristics and statistical data.

Parameters	Values
Maximum absorbance wavelength	432 nm
Linear range	10-50 µg.mL^{-1}
The equation of the calibration curve	A=0.0157 C - 0.004
Slope ± SD	0.0157±0.0003
Intercept ± SD	-0.004±0.007
LOD	1.47 µg.mL^{-1}
LOQ	4.41 µg.mL^{-1}

Accuracy: The accuracy of the developed method was calculated using the standard addition method. The results are shown in Table 2. The recovery of the added pure drug was calculated as, % Recovery-$[(C_t-C_s)/C_a]\times100$, where C_t is the total drug concentration measured after standard addition; C_s, drug concentration in the formulation sample; and Ca, drug concentration added to the formulation.

Table 2. Results for the determination of accuracy by analyzing sultamicillin in known concentration.

Concentration of drug in formulations, µg.mL^{-1}	Concentration of pure drug added, µg.mL^{-1}	Found, µg.mL^{-1}	Recovery, mean ± SD
30	15	45.23±0.74	100.46±1.62

Precision: Precision of the developed method was assessed as repeatability (intraday) and intermediate precision (inter day), by analysing 6 replicate determinations at three different concentrations. The results are shown in Table 3.

Table 3. Intraday and interday precisions of the method.

Concentration (μg.mL^{-1})	Precision (RSD%)
Intra-day	
10	1.69
30	0.70
50	1.89
Inter-day	
10	1.10
30	1.01
50	0.85

Pharmaceutical application

The proposed method was successfully applied to a sultamicillin pharmaceutical preparation. Sultamicillin 375 mg tablets were analyzed by the proposed method and the official method (Ph. Eur. 6th. edition). The results obtained from both methods were compared statistically by the Student's t-test and F-test in view of the precision and accuracy at a 95% confidence level. Calculated t and F values did not exceed the theoretical tabulated values, indicating no significant difference between the proposed and the official methods.

The mean percentage recovery, relative to the label claims, obtained by use of the proposed method, ranged from 101.3% to 101.6% (Table 4).

Table 4. Determination of sultamicillin in its pharmaceutical preparation by the proposed spectrophotometric and the official methods.

Pharmaceutical preparation	Mean ± SD		t	F
	Proposed method	Official method		
375 mg	380.38±1.26	380.16±0.90	0.31	1.96
n=6, P=0.05, t=2.23, F=5.05				

The proposed method is based on the derivatization of sultamicillin with NBD-Cl. This method has alower linear concentration range, LOD and LOQ values compared to the previously reported spectrophotometric method of Kshirsagar and et. all. (Kshirsagar et al., 2018). The standard deviations and recoveries of the two methods are close to each other. On the other hand, the derivatization and extractionsteps make the proposed method more selective than the previous UV specrophotometric method. So, the proposed method is superior to theprevious method in view of the sensitivity and selectivity for the determination of sultamicillin in pure, and its pharmaceutical dosage forms without any interference from excipients.

As it is seen in Table 5, the proposed method is superior to the previous method in view of the linear concentration range, LOD, LOQ and recovery. So, the proposed method is suitable for the determination of sultamicillin in pure, and its pharmaceutical dosage forms without any interference from excipients.

Table 5. Comparison of analytical performances of proposed and the previous methods.

Parameters	Proposed method	Previous method (Ph. Eur. 6th. Edition)
Linear range (μg. mL^{-1})	10-50	20-100
LOD (μg.mL^{-1})	1.47	3.67
LOQ (μg.mL^{-1})	4.41	11.18
Recovery (%)	100.46	99.50

CONCLUSION

This paper presents the development of a sensitive and simple spectrophotometric method to determine sultamicillin after its derivatization with a NBD-Cl reagent. Compared with previous methods, this method gave the highest sensitivity in measuring low sultamicillin concentrations. The suggested method might be used for routine sultamicillin analysis in dose forms. The method was validated and can be easily adapted for industrial analysis.

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

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Original Article

Development and validation of an HPLC method for determination of carbamazepine in human plasma and applications to a therapeutic drug monitoring study

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ABSTRACT

Background and Aims: Carbamazepine (CBZ) is an antiepileptic drug, which is prescribed as a first-line drug for the treatment of partial and generalized tonic–clonic epileptic seizures. The aim of this study was to develop and validate a simple, fast and reliable HPLC method for the determination of carbamazepine in human plasma.
Methods: Chlorpromazine (CPR) was used as an internal standard. The separation was conducted with a C18 reverse-phase column (150x3.9 mm, 5 µm) at 30°C, using a mobile phase prepared with 20 mM KH2PO4, acetonitrile and methanol (6:3:1, v/v/v) by isocratic elution.
Results: The method was linear between 0.5 and 40 µg/mL, determined by 10 individual calibration points. Total run time was ≤ 5 mins. Accuracy (RE%) values were determined between (-5.6) and 3.6%, and precision was determined at ≤4.2%. Limit of detection (LOD) was 0.04 µg/mL. The robustness test results of the method showed good values. Plasma CBZ of (n=30) those receiving CBZ quantities ranging from 0.2 to 1.2 g/day were measured with this method, and following analyses of their concentrations were found to be between 0.1 and 11.4 µg/mL (6.2±2.4 µg/mL). While all plasma sample analyses were applied properly, it was observed that 16 (53.3%) of the plasma samples had CBZ lower than the recommended range. In addition to that, female patient plasma-CBZ levels were found significantly higher than male plasma contents ($p<0.05$).
Conclusion: This method was found suitable for the analysis of plasma samples collected during the therapeutic drug monitoring (TDM) of patients treated with CBZ.

Keywords: Carbamazepine, plasma, therapeutic drug monitoring, validation, high-performance liquid chromatography, ultraviolet detection

INTRODUCTION

Carbamazepine (CBZ), (5H-Dibenz [b.f] azepine-5-carboxamide) (Figure 1) is used to treat bipolar disorder, especially (Chen & Lin, 2012) with geriatric patients' and individuals with multiple complaints (Punyawudho et al., 2012). But, it is extensively used for the management of epilepsy and also trigeminal neuralgia (Obermann 2010). In addition, it is also used as an anticholinergic agent. CBZ is considered a major antiepileptic drug which is used clinically to control different type of seizures. Its chemical structure is similar to tricyclic antidepressants. CBZ is a weakly acidic and non-polar aromatic ester. Since its chemical structure has a carbamoyl moiety at the 5th position (Figure 1), it is considered an iminostilbene derivative (Mittal & Das, 2012) which gives it anti-seizure activity.

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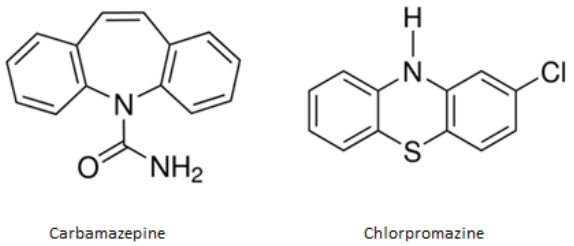

Figure 1. Chemical structures of CBZ and CPZ.

CBZ shows high oral bioavailability in humans (Marino et al., 2012). Since it is almost completely metabolized in the body, only a small part is excreted in the urine (Brunton, Knollman & Hilal-Dandan, 2018). The CBZ-10.11 epoxide is an active metabolite of CBZ, and also displays anticonvulsant properties similar to those of the parent compound (Chbili et al., 2017). Therapeutic concentrations of CBZ have been reported to range from 6 to 12 µg/mL (Brunton et al., 2018). CBZ is a potent inducer of the CYP3A4 and CYP2B6 enzymes, which are the main enzymes involved in the metabolism of this drug, and this fact is named auto-induction. Due to this property a significant decrease is observed in plasma drug levels during the first few weeks of ingestion (Ware, Tillery & Linder, 2016).

Thirty percent of the patients with focal epilepsy do not respond to a maximum dose of CBZ, so there is a need for additional anti-epileptic drugs to control their seizures, but it is also known that this combination treatment has the risk of the causing drug interactions. CBZ is used in combination therapy with other antiepileptic drugs, such as valproic acid or phenytoin, if necessary. The monitoring of blood drug levels is crucial to minimize the risk of toxicity while the CBZ treatment is in progress. Multidrug therapy may result in unexpected blood-drug levels due to changes in the activity of enzymes responsible for the metabolism of drugs (Datar, 2015). It is well known that geriatric patients are generally very susceptible to multidrug therapy because of the decrease in enzyme activities responsible for drug metabolism.

Several methods have been published for the determination of CBZ in pharmaceutical preparations and human specimens, including spectrophotometry (Frag, Zayed & Omar, 2012), FT-Raman spectroscopy (Farias & Carneiro, 2014), spectrofluorimetry, micellar electrokinetic capillary chromatography (MECC) (Datar, 2015), fluorescence polarization immunoassay (FPIA) (Sánchez, Garcia & Abadín, 2010), chemiluminescence (Leite, Petersen & Lunardelli, 2009) and gas chromatography-mass spectrometry (GC-MS) (Rani & Malik, 2012). Many of these methods are complex, expensive, time-consuming, and may require special sample preparation techniques and instruments.

HPLC is a simple, precise, accurate and cost-effective method and provides excellent recovery with high precision for a wide range of pharmaceutical compounds (Deeb et al., 2014; Domingues et al., 2016). It is very successful in the simultaneous determination of chemicals or metabolic products in biological samples. This technique allows the separation, identification and quantitative measurement of each component that forms a mixture. The method includes a liquid sample being passed over a solid adsorbent packed into a column using a flow of liquid solvent known as the mobile phase. The different interactions of each analyte in the sample with the adsorbent cause the flow of analytes in the column to change. If the interaction is strong, the analytes flow off the column for an extended period of time and if the interaction is poor, the elution time is short (Datar 2015).

Methods of detection of CBZ and its impurities by HPLC are reported in the United States Pharmacopoeia (USP), Euro-Pharmacopoeia (EP), British Pharmacopoeia (BP) and Indian Pharmacopoeia (IP). Some pretreatment techniques such as liquid-liquid extraction (Juhacik & Jenkins, 2009; Lajeunesse et al., 2009), solid-phase extraction (Bugamelli et al., 2002; Fortuna, Sousa & Alves, 2010; Franceschi & Furlanut 2005; Vermeij and Edelbroek 2007), solid phase micro extraction (Ferreira et al., 2009), stir bar-sorptive extraction (Queiroz et al., 2008), and deproteinization (Leite et al., 2009) have been used for the determination of CBZ and its metabolites by HPLC in plasma. Therefore, HPLC became prominent as a reliable technique for the detection of these and other anticonvulsant drugs (Bugamelli et al., 2002; Fortuna et al., 2010; Franceschi & Furlanut 2005; Dordević, Kilibarda & Stojanović, 2009; Mowafy, Alanazi & Maghraby, 2012; Queiroz et al., 2008; Ezzeldin, Shahat & Basudan, 2013; Vermeij and Edelbroek 2007).

The aim of our study was to develop a rapid, accurate and reliable HPLC method, with a simple, repeatable and inexpensive technique for the quantitative determination of CBZ in human plasma. In order to prove the applicability of real samples for this developed and validated method, it was planned to be used for determining drug levels in the plasma of epilepsy patients who are using CBZ daily.

MATERIAL AND METHODS

Chemicals and reagents

Chemical standards of CBZ and chlorpromazine (CPZ) (Figure 1), used as an internal standard, were obtained from Sigma (Steinheim, Germany). HPLC grade methanol and acetonitrile were ordered from Sigma-Aldrich (Missouri, USA). Orthophosphoric acid, potassium dihydrogen phosphate and triethylamine were purchased from Merck (Darmstadt, Germany). Membrane filters (0.45 µm pore size) used for filtration of mobile phase were obtained from Millipore (Massachusetts, USA). The Elga Purelab Water Treatment System was used to obtain ultra-pure water (Lane End, UK).

Instrumentation and chromatographic conditions

Agilent 1100 series HPLC system equipped with a degasser (G1322A), a gradient pump (G1311A, QuadPump), a manual injector (Rheodyne 7725i) with a 20 µL loop volume, a column oven (G1316A), and an ultraviolet detector (G1314A) was used in this study. The analytical separation was performed by a stainless steel C_{18} analytical column (150 mm x 3.9 mm I.D. 5 µm p.s.) packed with ODS particle-NovaPak (Waters, Japan).

Optimum analytic conditions were set after an optimization procedure was performed for the column selection, content

of mobile phase and wavelength. Prior to optimization, an assay for the CBZ determination was chosen using the literature data, and each parameter was adjusted or fixed. UV detection was adjusted to 220 nm. The mobile phase was prepared with 20 mM phosphate buffer (0.1% triethylamine, pH 3.0), acetonitrile, and methanole (60:30:10, v/v/v). Before each use, the mobile phase was filtered and then degassed in the ultrasonic bath for 30 minutes. The mobile phase solution was isocratically applied to the column by 1.0 mL/min flow rate, at 30°C.

The unknown concentrations of CBZ were quantified using linear regression of response (drug/ISTD peak area) versus CBZ concentrations. Chemstation®, 08.03 version (California, USA) was used for the control of system equipment and data integration.

Preparation of chemical standards

Main stock solutions of CBZ (2 mg/mL) were prepared in methanol. The working solutions of CBZ were prepared weekly from the main stock solution with methanol as 25, 50, 100, 250, 500 and 1000 µg/mL concentrations. Working standards were prepared weekly, and used to spike blank plasma samples daily, prior to analysis of freshly prepared CBZ dilution in drug-free human plasma to provide concentrations of 0.5, 1, 2, 5, 10 and 20 µg/mL. Fresh human blood samples were collected, to act as a negative control, from the Faculty of Medicine Blood Center. The blood was centrifuged at 3000 rpm for 5 min to separate the plasma. Plasma samples, and all the working solutions were stored at -20˚C until the analyses were carried out. Working solutions were checked chromatographically for purity before the experiments, utilized as quality control specimens and were checked for stability before and after the injections of every sample set.

The ISTD main stock solution (1 mg/mL) was diluted weekly with methanol to yield a 100 µg/mL working solution. Plasma quality control standards, spiked with 1, 5 and 20 µg/mL of CBZ, were prepared to measure the repeatability values of the method. The same protocol was used in the preparation of the limit of detection (LOD), quantification (LOQ), robustness, recovery and stability samples.

All standards were stored at -20°C until use. It was observed that they were stable for at least 1 month.

Sample preparation

As an ISTD, 10 µL CPZ (100 µg/mL) and 10 µL CBZ as a working solution (performed on validation test samples only) were both added into the 250 µL of blank plasma sample, and then 200 µL methanol was added. After the sample tube was mixed by vortex at 1200 rpm for 2 min, it was centrifuged at 10000 rpm for 7 min to precipitate the plasma proteins. Finally, the upper clear phase was collected and loaded to the liquid chromatograph as 20 µL volume manually.

Selection of ISTD

In order to determine the ISTD; fluphenazine, opipramol, imipramine, sildenafil, and CPR were tested. Some of the tested chemicals did not demonstrate the acceptable ultraviolet intensity, and some did not show appropriate retention times. Finally, CPR was selected as the ISTD as its separation sharpness and retention time was acceptable in the chromatogram. In addition, CPR showed a very good intensity in low concentrations. The obtained extraction recovery values were shown to be acceptable and reproducible for ISTD.

Method validation

The developed chromatographic technique was validated for selectivity, linearity, accuracy, precision, the limit of detection (LOD) and limit of quantification (LOQ), recovery and robustness. In agreement with theInternational Conference on Harmonization (ICH) guideline, the intraday and inter-day validation protocol were applied considering the reproducibility of method in order to obtain accurate and precise measurements (ICH, 2005).

Selectivity

The method showed good chromatographic (in plasma matrix) without interference at the retention times of 2.6 and 4.0 min for CBZ and CPZ respectively. Representative chromatograms of blank plasma (Figure 2a), spiked plasma (Figure 2b) and patient plasma samples (Figure 2c) illustrate the high reso-

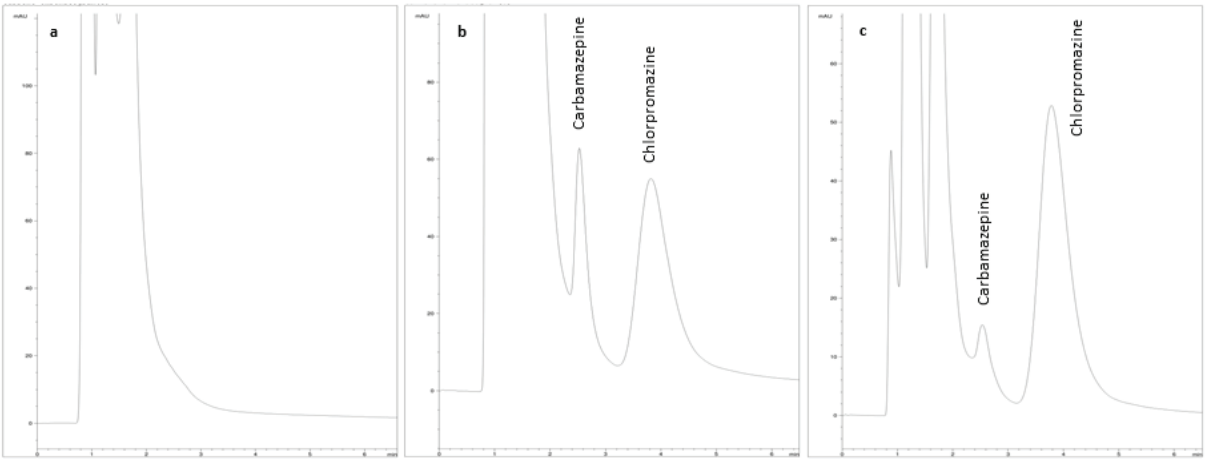

Figure 2a. The chromatogram of blank plasma used in validation tests. **b.** The chromatogram of plasma sample that contained CBZ as 5 µg/mL which is prepared by standard addition method used as quality control sample. **c.** The chromatogram of real patient plasma sample.

lution in a short 6 minute separation time. Although the 210, 214, 237 and 285 nm ultraviolet values were recommended in the literature, the highest peak yield for both the internal standard and analyte was obtained from 220 nm. Therefore, the ultraviolet detector (UV) was set to 220 nm.

Linearity

After chromatographic conditions were established, matrix-based calibration curves of CBZ were plotted over the range 0.5 - 40 µg/mL versus peak-area ratios to the ISTD. The calibration points (n=7), which were 0.5, 1, 2, 5, 10, 20 and 40 µg/mL composed of 3 individual replicates, were prepared by the standard addition method in plasma and injected into HPLC.

Accuracy and precision

The accuracy, defined as the relative error (RE%), was calculated as the percentage difference between the added and found CBZ quantity by 5 separate replicates both intraday and inter-day. The precision, defined as relative standard deviation (RSD%), was calculated by five separate replicates of CBZ both intraday and inter-day. Five replicate spiked samples were assessed both intraday and inter-day at the three different concentrations (1, 5 and 20 µg/mL).

Sensitivity

The concentration of 0.5 µg/mL, as the lowest calibration point, was used in the sensitivity tests of CBZ. Individually, 10 quality control (QC) samples were prepared on the same day and applied to sequential analyses. Obtained chromatograms' analyte peaks belong to CBZ and CPZ were calculated with calibration data prepared in the linearity test.

Recovery

The recovery of extraction procedures from human plasma was determined by comparing pre-extraction spikes with the post-extraction spiked ISTD. Five individual replicates of spiked samples at low, middle and high concentrations (1, 5, 20 µg/mL, respectively) of CBZ were prepared with and without ISTD. The extraction procedure was carried out as described in the sample preparation step.

Robustness

The response of the method of changes in UV wavelength (±3 nm), mobile phase flow rate (±0.1 mL/min), mobile phase organic solvent content (±5%) and column temperature (±4°C) was observed.

Collection of plasma samples

1.5 mL of whole blood samples were taken from the patients which had a steady-state concentration of CBZ in the plasma. Generally, blood samples were taken from patients at 9.00 a.m. in the morning. The vacuum tube containing Na_2EDTA was used for collection of the samples. The whole blood was centrifuged at 3500 rpm for 5 minutes, and the plasma obtained was stored at -18°C until analyzed.

The volunteer blood samples were obtained from 30 patients who were under the CBZ treatment at the Medical School, Department of Neurology. Plazma CBZ levels were measured within 15 days after blood samples were taken from the patients.

Statistical analysis

All statistical analyses were done using the IBM SPSS software version 22. The analysis of plasma-CBZ data produced from the developed method was performed by Independent-samples T test. Paired-sample T test was used for comparison of the plasma-CBZ results, which were obtained by two different analysis methods. The statistical significance level was accepted as $p<0.05$ for all analyses performed. Results were presented as the mean±SD.

RESULTS AND DISCUSSION

Linearity

The calibration curve of CBZ was drawn by the standard addition method at 7 points (n=3) between 0.5-40 µg/mL concentration versus the area of CPZ as ISTD. The obtained correlation of coefficiency was calculated as r^2=0.9983, (Table 1). The linearity study was designed to cover sub-therapeutic, therapeutic, overdose and toxic levels of the CBZ in plasma.

Table 1. Chromatographic characteristics and system suitability parameters of the method.

Analyte	Wavelength (nm)	Retention time TR (min)	LOD (µg/mL)	LOQ (µg/mL)	Linear Range (µg/mL)	Calibration equation	Correlation coefficient (r^2)	Capacity factor (k')	Theoretical plate number
CBZ	220	2.6	0.040	0.122	0.5-40	y=0.0519x+0.023	0.9983	1.6	3972
CPR	220	4.0	-	-	-	-	-	2.8	

Sensitivity

The limit of quantification (LOQ) and limit of detection (LOD) were calculated according to the ICH recommendations based on standard deviation of the response and the slope of the calibration graph. Equations were given as: LOQ= 10σ/S; LOD= 3.3σ/S (σ: The standard deviation of the response; S: The slope of the calibration curve) (ICH, 2005).

The results of LOD and LOQ values are shown in Table 1. The obtained LOD and LOQ values were sufficient for both identification and quantitative analysis of CBZ.

Precision and accuracy

The data obtained from the accuracy and precision tests (Table 2) performed in intraday and inter-day with quality control standards established in the blank plasma samples by standard addition method showed a low RSD% value ≤4.2 and also low RE% values between (-5.6%) and 3.6%. The obtained intraday and inter-day repeatability values support that the method can be applied safely in real blood samples.

Table 2. Confidence parameters of the method that including intraday and inter-day precision and accuracy.

Conc. (µg/mL)	Intraday				Inter-day				Average Recovery (%)
	No. Obs.	Estimated conc. $\bar{X}$±SD (µg/mL)	Precision (RSD%)	Accuracy (RE%)	No. Obs.	Estimated concen. $\bar{X}$±SD (µg/mL)	Precision (RSD%)	Accuracy (RE%)	
1	5	0.96±0.03	3.6	-3.7	5	0.94±0.04	4.2	-5.6	82.4
5	5	4.84±0.13	2.8	-3.2	5	4.85±0.18	3.8	-2.8	88.4
20	5	20.69±0.26	1.3	3.6	5	20.60±0.39	2.0	3.0	105.7

Recovery

Recovery test results were between 82.4% and 105.7% (92.2% ±12.1), and are given in Table 2. The recovery values that were obtained in the extraction procedure, demonstrated efficiency. It was observed that the extraction procedure was not complicated and did not require a sophisticated instrument to carry out the method. The recovery data of this method was reliable, and was produced after firstly carrying out protein precipitation followed by sample the preparation protocol based on HPLC application.

Robustness

No significant changes were observed in the analytical signals upon changing the UV wavelength value (±3 nm), mobile phase flow rate (±0.1 mL/min), mobile phase organic solvent ingredient (±5%) or column temperature (±4°C). Not only changes in analysts, columns, sources of chemicals and/or solvents, but did not lead to significant changes in chromatographic signals. Robustness experimental results demonstrated that the method is able to create data with acceptable precision and accuracy. Changing the analysts, columns, and sources of chemicals/solvents did not lead to significant changes in chromatographic signals.

Stability

The stability of QC samples (1, 5 and 20 µg/mL) and analytes in the stock solutions of the analytes were assessed under several conditions. Firstly, the stability of the stock solutions of CBZ and CPR were evaluated at the end of each week for 4 weeks, using the same analysis method. In this test, stocks were stored at 4°C. A stability test for freeze-thawing was executed for three QC samples (1, 5 and 20 µg/mL of CPZ) after carrying out five repeated freeze-thaw actions. The stability test for long-term CPZ storage was carried out at 1, 2 and 3 months using QC samples kept at -20°C. Neither a significant decrease nor degradation was observed in the concentration of CBZ in the different storage times. The relative standard deviation (RSD) was less than 4% for all samples.

There are some studies for analysing CBZ levels in blood samples. In the HPLC-based study performed by Mowafy et al. (2012), a new CBZ determination method was developed and validated for rabbit plasma (Mowafy et al., 2012). In this study, separation was performed with µ-Boundpak C18 (150 mm x 4.6 mm i.d.) column by mobile phase consisting of methanol and water (1:1, v/v. Propylparaben was used as an internal standard. Detection was accomplished by a UV detector set at 285 nm. The retention time of CBZ and propylparaben were 7.7 and 11.4 min, respectively. Total run time was 15 min . Intra day and inter-day accuracy were reported between 97.5 to 103.6%. Intraday and interday precision were found to be ≤3.7%. The calibration curve was found to be linear in the range of 0.5–40 µg/mL.

In the study conducted by Vermeij & Edelbroek (2007), the plasma levels of 7 antiepileptic drugs were analyzed simultaneously, using the HPLC-DAD method. Acetonitrile, methanol and phosphate buffers were used as mobile phase. A 150 mm x 4.6 mm C18 analytical column was used for analytical separation. The calibration range is 0 to 14.8 µg/mL. The retention time of CBZ was 18.3 minutes, and the total analysis time was 20 minutes. A volume of 0.1 mL serum sample was extracted by solid phase extraction. The sample volume loaded onto HPLC after extraction was 50 µL. The intraday precision was <1.9% and the LOQ was <0.065 µg/mL.

In the HPLC-UV-based study performed by Franceschi & Furlanut (2005), 3 different antiepileptic drugs and 2 metabolites with CBZ were observed in the blood (Franceschi & Furlanut 2005). The solid phase extraction method was used, and analysis was performed with 0.5 mL volume of serum. The UV detector is set to 214 nm. Cyeptamide was used as an internal standard. The 250 mm x 4.6 mm analytical column was used for separation. Water, acetonitrile, methanol, acetic acid and triethylamine (72.5:15:125/0.1/0.06, v/v/v/v/v) were used as the mobile phase. The flow was 1.2 mL / min and the retention time of CBZ was 10.5 min, so the total analysis time was 15 minutes. Between 0 - 60 µg/mL CBZ was used for calibration. The accuracy was determined between 99 and 105%. The LOQ was 0.2 µg/mL, and the recovery was determined between 95.78 - 102.84%.

An HPLC-based method was developed by Bugamelli et al. (2002) to determine oxcarbamazepine, CBZ, lamotrigine, phenobarbital, primidone, phenytoin and two metabolites in human plasma. The total analysis time was 11.5 minutes. The C18 column (150×4.0 mm, i.d. 4.5 µm) was used for separation. The mobile phase was 15 mM phosphate buffer (0.63% triethylamine), methanol and acetonitrile (64.0: 19.2: 16.8, v/v/v). The flow rate was 1 mL/min, and the DAD detector was set at 237 nm. Plasma samples were first precipitated with perchloric acid, and then

extracted using the solid phase method. The total analysis time was 13 min, and the retention time of CBZ was 11.5. minutes. The LOQ was 60 ng/mL. The recovery was 91%. Precision was determined between 8.1 and 5.0%.

Fortuna et al. (2010) developed an HPLC-UV method for determining the concentrations of CBZ, oxcarbamazepine, eslicarbazepine acetate and metabolites in human blood. A C18 column (55 mm×4 mm) was used for separation. Water, methanol and acetonitrile (64:30:6, v/v/v) mobile phase flow rate was 1 mL/min, and the column temperature was 40°C. Total analysis time was 9 min, and the retention time of CBZ was 7 min. The linearity test was performed between 0.05-30 µg/mL. The accuracy was 12%, the LOQ was 0.05 µg/mL and the precision was 8.68% (RSD%). The ultraviolet detector was set at 235 nm.

Dordević et al. (2009) established a new HPLC-UV method for CBZ in saliva and serum. Separation was achieved by a reversed phase C_{18} column with a mobile phase of methanol, water and acetic acid (65:34:1, v/v/v). Flow was 1.0 mL/min. UV was set at 285 nm. In the method, the liquid-liquid extraction method was used for the extraction of alkalinized samples with chloroform. Total run time was 5 min. The method was linear in the range of 0.1–5 µg/mL for both types of sample. The average recovery for serum was found as 97.59%. The method precision for serum was found between 2.10% to 4.03%. LOQ of the method was detected as 0.237 µg/mL for serum sample. Method accuracy was found at 0.52.

Queiroz et al. (2008) developed an HPLC-UV method for the monitoring CBZ, its main metabolite, phenytoin and phenobarbital in human blood samples. Chromatographic separation was performed using the C8 column, the UV detector was adjusted to 210 nm. The calibration range was 0.08 - 40 µg/mL. The LOQ was 0.125 µg/mL. The accuracy was between 3.3 and 9.2%.

Ezzeldin et al. (2013) described an HPLC-UV method for the determination of CBZ in human plasma. Diclofenac was used as an internal standard. Separation was conducted by a C8 (250 x 4.6 mm, 5 µm) column with a mobile phase prepared with buffer (pH:3.0), acetonitrile and isopropyl alcohol (49:36:15, v/v/v). Chromatographic determination was achieved at 220 nm. LOQ was 0.1 µg/mL. Total run time was 20 min. Precision was ≤12.8%. Intraday and interday accuracy were determined between 85 to 100%. Average recovery was 87.0%. All these methods, published in the literature, were summarized in Table 3.

Table 3. The validation parameters and chromatographic properties of CBZ determination methods published in literature.

Study	Instrument	Linear range (µg/mL)	LOQ	Recovery (%)	Mobile phase	Flow (mL/min)	Retention time (min)	Total analysis time (min)	Detection wavelength	Column	Precision (RSD%)	Accuracy	Column oven temperature (°C)
Franceschi & Furlanut, 2015	HPLC-UV	0–60.0	2.0 µg/mL	95.8-102.8	Water, ACN, MeOH, Acetic acid, TEA (725:150:125: 0.1, 0.06, v/v/v/v/v)	1.2	10.5	15.0	214 nm	CN, 250 mm x 4.6 mm, 5 µm	-	99.0 - 105.0 %	50.0
Bugamelli et al., 2002	HPLC-DAD	2–40 µg/mL	60.0 ng/mL	91.0	PB, TEA, MeOH, ACN (64.0: 19.2: 16.8, v/v/v)	1.0	11.5	13.0	237 nm	C18, 150 mm x 4.0 mm , 4.5 µm	5.0 - 8.1	-	-
Fortuna et al., 2010	HPLC-UV	0.05–30.0 µg/mL	0.05 µg/mL	80.0–91.9	Water, MeOH, ACN (64:30:6, v/v/v)	1.0	7.0	9.0	235 nm	C18, 55 mm x 4.0 mm	8.7	12.0 (RE%)	40.0
Dordević et al., 2009	HPLC-UV	0.1–5 µg/mL	0.2 µg/mL	95.2–106.0	MeOH, water, acetic acid (65:34:1, v/v/v)	1.0	3.9	5.0	285 nm	C18, 250 x 4.0 mm, 5 µm	2.1 – 4.0	0.5 – 1.9 (RE%)	-
Queiroz et al., 2008	HPLC-UV	0.08–40.0 µg/mL	0.08 µg/mL	72.0–86.0	Water: ACN (78:22, v/v)	1.0	32.0	40.0	210 nm	C8, 125 mm × 4 mm, 5 µM	8.8 – 10.0	3.3 – 9.2 (RE%)	-
Ezzeldin et al., 2013	HPLC-UV	0.1–8.0 µg/mL	0.1 µg/mL	87.0	ACN: IPA: PB (36:15:49, v/v/v)	1.2	11.4	14.0	220 nm	C8 250 x 4.6 mm, 5 µm	4.2 - 12.8	85.0 – 100.0 %	-

ACN: Acetonitrile, MeOH: methanol, TEA: Triethylamine, IPA: isopropyl alcohol, PB: phosphate buffer

CBZ is susceptible to temperature changes, so its analysis by gas chromatography is not suitable. For this reason, before analysis by gas chromatographic method, it is derivatized with some agents (Minkova & Getova, 2001). In the study conducted by Auer et al. (2003), carbamazepin stability was observed in a commercial dosage form. As a result of this study, it was found to be stable. Matar et al. (1999) investigated the stability of CBZ in their HPLC based analysis study, which was conducted after being stored 4 weeks at -20°C, and according to obtained result, CBZ was found to be stable (Matar, Nicholls & Tekle, 1999). Tonic-Ribarska et al. (2012) investigated the stability of CBZ in their study, and they conducted this study on stock solutions of CBZ in 24 h at room temperature and 3 months at 2–8°C, and they found it as stable (Tonic-Ribarska, Haxhiu & Sterjev, 2012). In our study, CBZ and CPR as internal standards were clearly observed to be stable at +4°C for 4 weeks, and at -20°C for 3 months. In the stability test applications, no degradation or decrease was observed in the areas of peaks of CBZ and CPR.

We have developed and validated the application in 30 epilepsy patients with blood samples as well as the practical application of our study. It was observed that the validation data contains efficient results. It has been found that the calibration range of 0.5 to 40 µg/mL can be used successfully at different concentration levels of CBZ. The reproducibility test results of the method performed at 3 different concentration points were found to be reliable when compared to the methods in the literature. The accuracy was ≤4.2% during the same day and between days. The accuracy was between 5.6 and 3.6. Lower retention times than in the literature were obtained using a 150 mm C_{18} column. İt is a simple, fast and reliable analysis method with high recovery values obtained with this protein precipitation method. This is an advantage of the method as it allows for routine analysis. Plasma (250 µL) was used in the analysis. The resulting LOQ value was 0.122 µg/mL. This value is below the calibration range, so it proves that the method is suitable for highly sensitive analyses.

Patient plasma CBZ levels

CBZ levels in blood samples taken from patients receiving CBZ oral treatments as 200, 300, 400, 600, 800 and 1200 mg/day for epilepsy treatment were monitored with the developed and validated HPLC method. Analyses were performed with a 0.25 mL plasma sample taken from patients with a steady-state concentration of the drug in their blood. None of these samples showed any problem for the quantification of the analytes. Additionally, peak purity showed that no analytical interference was encountered from endogenous substances. The obtained blood CBZ level results are given in Table 4 and Table 5, and comparision of the CBZ blood levels results and the dose rated blood levels of CBZ results were represented in Figure 3. CBZ was observed to be dose-dependent among individuals in the plasma samples of 30 patients. The presence of CBZ in plasma in a dose-dependent manner is also an indication that the drug is suitable for TDM.

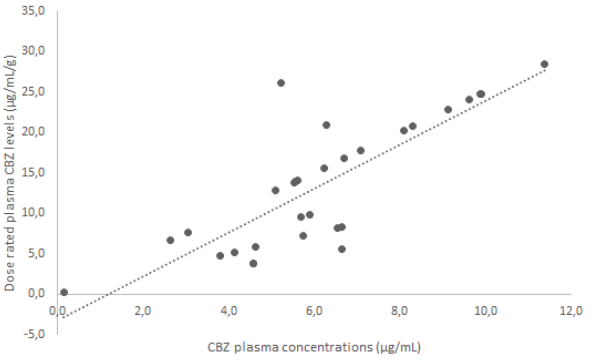

Figure 3. Comparision of the CBZ blood levels results and the dose rated blood levels of CBZ results.

Recommended blood plasma values for the treatment with CBZ is 6 and 12 µg/mL (Rani & Malik, 2012). The results of the study showed that 16 out of 30 patients treated with CBZ (53.3%) had blood concentrations below these values. This result showed that more than half of the patient population followed up did not reach an effective blood concentration, and continued their treatment. However, this is very important because the patients show that they continue to undergo treatment for CBZ at a lower level than recommended. This means that, despite drug intake, serious symptoms of the disease cannot be prevented.

Table 4. Number of samples included in the analysis, daily CBZ doses, serum concentrations and dose-based blood results.

Pateint number	Dose (mg)	Plasma result (µg/mL)	Plasma result / Dose	Pateint number	Dose (mg)	Plasma results (µg/mL)	Plasma result / Dose	Pateint number	Dose (mg)	Plasma results (µg/mL)	Plasma results / Dose
1	400	5.2	13.1	11	400	5.6	13.9	21	400	5.7	14.2
2	400	6.3	15.7	12	400	3.1	7.6	22	400	5.7	14.3
3	800	5.5	6.9	13	1200	9.9	8.2	23	400	6.5	16.3
4	1200	9.6	8.0	14	400	2.6	6.6	24	400	3.8	9.5
5	800	11.4	14.2	15	400	6.2	15.6	25	300	4.6	15.4
6	1200	5.6	4.7	16	800	9.9	12.4	26	800	6.6	8.3
7	800	8.3	10.4	17	800	9.1	11.4	27	200	4.1	20.7
8	600	7.1	11.8	18	400	6.7	16.7	28	400	4.6	11.5
9	400	8.1	20.3	19	600	5.9	9.8	29	400	6.7	16.6
10	400	5.1	12.8	20	600	0.1	0.2	30	400	4.6	11.4

Table 5. Descriptive statistics of real plasma results obtained from two different methods.

	1st metod results		2nd method results	
	Plasma result (µg/mL)	Plasma result (µg/mL)/Dose (g)	Plasma result (µg/mL)	Plasma result (µg/mL)/Dose (g)
Average	6.2	12.0	6.1	13.5
SD	2.4	4.5	2.2	8.0
RSD%	38.8	37.7	36.7	59.5

Abbrevisions: 1st method: Our developed analysis method, 2nd method: Analysis method routinely used by the hospital

Plasma concentrations of CBZ are significantly lower in polytherapy than in monotherapy (Koristkova, Bergman & Grundmann, 2006). The use of phenytoin and some barbiturates with CBZ in the treatment of epilepsy is frequently encountered in the clinic. This combined treatment (polytherapy) results in a significant auto-induction of CYP450 enzymes. This situation results in a decrease in plasma CBZ levels. Pregnancy is an important period that seriously affects the activity of CYP450 enzymes. During this term, plasma CBZ levels decrease, possibly due to increased activation of all the metabolic enzymes (Bertilsson, 1978). CYP3A4, which is responsible for the metabolism of more than 50% of therapeutic drugs, and also CBZ, show higher activity in women than in men. However, there are also studies reporting that there is no gender difference in the study rates of CYP enzymes. Many other CYP (CYP2C19, CYP2D6, CYP2E1) isozymes and conjugation (glucuronidation) activity, which is also so important, are reported to be higher in males than in females (Tanaka, 1999). According to this interpretation, drugs metabolized by these enzymes are observed in the plasma of women at a higher concentration than in men (Tanaka, 1999). Although smoking and alcohol consumption are more common in men, drug metabolism is severely affected by gender-specific factors (menopause, pregnancy and menstrual cycle) in women. It is thought that the factors given above may be the reason behind the significantly higher CBZ plasma levels of women compared to men.

Also, drug-drug interactions and diet can affect the plasma CBZ levels. For example, vigabatrin decreases the plasma concentration of CBZ by increasing its clearance, not catabolism (Sánchez-Alcaraz, Quintana & López, 2002. Topiramate interferes with the plasma level of CBZ (Grunze et al., 2001). In some patients., zonisamide may increase CBZ serum levels. Topiramate clearance was 70% higher in patients treated with CBZ, and was found to increase with age (Mimaki, 1998). However, not all interactions are pharmacokinetic. CBZ plus stiripentol (a newer anticonvulsant) interacts pharmacodynamically, and its benefits may outweigh the usual disadvantages of polytherapy. As expected, fluvoxamine, an inhibitor of CYP4503A4, significantly increases plasma CBZ levels (Cottencin et al., 1995). However, fluoxetine, metabolised primarily by CYP2D6, does not interact with CBZ (Sproule, Naranjo & Brenmer, 1997) Similarly, pomegranate juice, which inhibits cytochrome P4503A4, significantly increases the AUC of CBZ in rats (Misaka et al., 2011).

It was thought that the effects of CBZ on CYP3A4 and CYP2B6 were important factors for the emergence of the results, that we got from the study. Also, individual metabolism rate differences observed in CYP3A4 and CYP2B6 enzymes are considered as important factors affecting the CBZ metabolism rate. These factors are capable of changing blood CBZ levels. Our study is related to the fact that female CBZ levels are significantly higher than male results, and that sex-specific factors (menopause, pregnancy and menstrual cycle) are significantly effective in CBZ metabolism.

The CBZ levels in the blood samples of 30 patients included in our study were analyzed by an immunoassay based approach. In this method, called cloned enzyme donor immunoassay (CEDIA), 1 mL of serum or plasma sample can be used in the analysis. Reported analytical measuring range was 0.5-20 µg/mL. This control analysis is a routine periodic application, and is performed at the initial stage of drug treatment or at the dose increase or decrease stages of the drug. With this analysis method, the plasma CBZ concentration can be determined in 1 hour in total, with other blood biochemical analyzes performed simultaneously. This follow-up may also be needed during combined drug therapy or because of complaints related to epilepsy.

Plasma-CBZ analysis results performed by the Faculty of Medicine were compared statistically with the results obtained by our suggested method. No significant difference was found between the results of blood drug levels obtained from both methods ($p>0.05$). Blood CBZ results from the two methods were rearranged according to the drug doses administered to the patients, and no significant difference was observed in the results from the two methods ($p>0.05$).

CONCLUSION

This HPLC method, which was developed and validated for quantitatif analysis of CBZ, was simple, rapid and reliable. The precision and accuracy test results of the method, which are RSD% ≤4.2 and RE% value were between (-5.6) and 3.6 respectively, showed good repeatability. Sample extraction was simple, rapid and provided reliable recovery values between 82.4% and 105.7%. Due to the simplicity of the sample preparation, the short analysis time (<6 min) and the high sensitivity, this technique was ideal for the quantification of CBZ in human plasma.

We recommend this validated method to be used in routine therapeutic drug analysis of CBZ, and it could be adapted for monitoring overdose/poisoning with this drug in suicide cas-

es. Also, this method can be directly applied in routine TDM studies of CBZ. It can be utilized in bioequivalence, pharmacovigilance and pharmacokinetics studies.

Although the recommended blood values in the CBZ treatment are 6-12 µg/mL, low drug levels observed in the blood values of the volunteers showed that it is necessary to perform TDM by a reliable method for the treatment of CBZ. Because of the significant difference ($p<0.05$) observed in drug plasma levels in patient plasma CBZ levels, the determination of polymorphism rates of CYP3A4 and CYP2B6 subtypes responsible for CBZ metabolism was planned as future work of this study.

Ethics Committee Approval: The ethical permission protocol was approved by The Local Ethics Committee with 2018-01/20 decision number on 10th January 2018. It was conducted in accordance with the Declaration of Helsinki and its subsequent revisions. Information consent forms were obtained from the volunteers before being included in the study.

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Original Article

Core shell column in high-performance liquid chromatography for the determination of polar compounds; troxerutin and carbazochrome

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ABSTRACT

Background and Aims: The combination of troxerutin and carbazochrome has been found efficacious for non-surgical patients with acute uncomplicated hemorrhoids. Therefore it is essential to develop a simultaneous analysis method of those drug substances. This study explains a simple and selective HPLC method to analyze troxerutin and carbazohrome in tablets.
Methods: The analysis was carried out using a core-shell pentafluorophenyl propyl polar column with a mobile phase consisting of (MeOH; % 5 HAc), (99.5:0.5 - V/V) at a flow rate of 0.25 mL/min. UV detection was set to 350 nm.
Results: The standard calibration curve was established between 1.00-20.00 µg/mL for troxerutin; 0.05-1.00 µg/mL for carbazochrome. LOD and LOQ were found to be 0.65 µg/mL and 1.00 µg/mL for troxerutin and 0.01 µg/mL and 0.05 µg/mL for carbazochrome, respectively.
Conclusion: The proposed method was validated for specificity, linearity, accuracy, precision, LOQ and LOD and then successfully applied for the analysis of these substances in tablets.

Keywords: Troxerutin, carbazochrome, high performance liquid chromatography, core-shell column, tablet

INTRODUCTION

Hemorrhoids, a pathologic dilatation of the hemorrhoidal venous plexus, is a common clinical problem world-wide. Venous insufficiency, of which hemorrhoids is one type, is an underestimated public health problem and can have a serious impact on patients' quality of life. The underlying cause of hemorrhoids can be treated with venotonic agents. It has been found that flavonoids, such as troxerutin, are not only effective but also safe agents for the treatment of chronic venous insufficiency (Basile et al., 2001). Troxerutin (TROX) is a trihydroxyethylrutin (Figure 1). It has antithrombotic, antierythrocytic, fibrinolytic, oedema-protective, andrheological activity and has been used therapeutically to treat chronic venous insufficiency, varicose veins and haemorrhoids. Troxerutin significantly inhibits platelet adhesion to the extracellular matrix, yields an anti-erythrocyte aggregation effect and exerts a favorable action on the blood fibrinolytic system. This is the reason for its positive effect on capillary perfusion, stasis and trophic complications of chronic venous insufficiency (Basile et al., 2001; Cui et al., 2011). Chemical formula of Carbazochrome (CARBO) is 5,6-dioxo-3-hydroxy-1-methyl-2,3,5,6-tetrahydro-1H-indole 5-semicarbazone (Figure 1). It is an adrenochrome derivative and has been widely preferred for treatment of hemorrhage due to the fragility of capillaries. It has been shown that carbazochrome can decrease the pulmonary dysfunction and vascular hyper-permeability (Song et al., 2010). In patients with chronic venous insuf-

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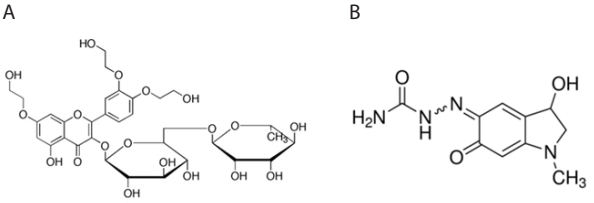

Figure 1. Structures of troxerutin (A) and carbazochrome (B).

ficiency, this combination of drugs has been proved to have a good efficacy and well tolerated (Squadrito et al., 2000).

Several methods were reported on each drug individually. In the literature, high-performance liquid chromatography (HPLC) methods (Joseph & Deepthi 2015; Hepsebah, Nihitha & Kumar, 2014; Yang et al., 2007), spectrophotometric method (Chen et al., 2015) and capillary electrophoresis method (Guo, Ron, Bı, & Sun, 2004) have been reported for analysis of troxerutin. Carbazochrom was examined using spectrophotometry (Gan et al., 2012; Wu & Liu, 2008), HPLC (Hu et al., 2014) methods, and the chemiluminescence method (Wang et al., 2011). Khattab et al. (2015) and Abdelrahman et al. (2016) determined that carbazochrome and troxerutin were from injectable pharmaceutical forms simultaneously.

The ability to retain and separate polar, hydrophilic molecules in reverse phase chromatography can be challenging and problematic. Separation generally requires the use of ion pair reagents, mobile phase and pH modification. C18 column has been used as a stainonary phase for HPLC methods in the literature (Joseph & Deepthi, 2015; Hepsebah et al., 2014; Yang et al., 2007; Hu et al., 2014; Abdelrahman, Abdelaleem, Ali & Emam, 2016). However we chose the core-shell pentafluorophenyl propyl polar column (PFP) to separete substances efficiently. Nowadays, the use of core shell technology has become increasingly popular to provide efficient separation and good peak shape in chromatography. Due to the core-shell particles, rapid mass transfer occurred and broadening from longitudinal diffusion, known as B term in the Van Deemter equation, was reduced (Hayes et al., 2014). The core shell particles column technology has gained a position of primary importance in HPLC due to advantages of resolution power.

The aim of this study is to develop a simple, safe method for the determination of the polar compounds based on UV- absorption detection with high performance liquid chromatography technique using core-shell PFP column and application of this method on tablet pharmaceutical preparations.

MATERIALS AND METHODS

Apparatus and reagents

The HPLC system of Agilent Infinity 1260 Series with UV detector were used for analysis of troxerutin and carbazochrome. The HPLC system consisted of 1260 Quat pump, 1260 ALS, 1290 Thermostat, 1260 TCC, 1260 DAD. Chemstation was used as instrument soft-ware.

Troxerutin and carbazochrome were obtained from World Medicine, Turkey with their certificates. Methanol and acetonitrile, which are HPLC grade, were purchased from Merck. Labels of acetic acid (100%) and analysis grade dimethyl sulfoxide were also obtained from Merck.

Chromatographic conditions

Chromatographic separation was carried out at a temperature of 25°C using a Phenomenex Kinetex Core-shell Pentafluorophenyl Propyl, 2,6 µm, 4,6x150 mm column. The mobile phase consisted of (MeOH:%5 HAc)(99.5:0.5-V/V) (v/v). The flow rate of the mobile phase was 0.25 mL/min. The detector was set at 350 nm. The injection volume of 5 µL was chosen.

Preparation of standard solutions and quality control samples

The stock solutions of troxerutin were prepared in ultrapure water (0.5 mg/mL) and diluted with methanol (100 µg/mL). The stock solutions of carbazochrome were prepared in dimethyl sulfoxide (0.5 mg/mL) and also diluted with methanol (5 µg/mL). All solutions were stored at 4°C until the end of the study. The five calibration standard containing different ratios of TROX (1-20 µg/mL) and CARBO (0.05-1.00 µg/mL) were prepared with methanol. The concentrations of quality control (QC) samples were at 1.00; 3.00; 15.00; 20.00 µg/mL for troxerutin and 0.05; 0.30; 0.70; 1.00 µg/mL for carbazochrome. All calibration standards and QC samples were stored at 4°C until the end of the study.

Preparation of tablet samples

Ten tablets containing 300 mg TROX and 3 mg CARBO were powdered and ¼ tablet weight was diluted in a 50 ml flask to volume with dimethyl sulfoxide, 6 replicates were prepared in this way. Recovery samples were also prepared by spiking drug substances in to plecoba at the same concentration. Further dilution was carried out by transferring the appropriate amount in methanol for 15.00 ug/mL for TROX and 0.15 ug/mL for CARBO final concentration.

Evaluation of the analytical method

The method development process is influenced by the nature of the analytes. The main part of this process is the selection of column and mobile phase composition. In this study, different column types and mobile phase compositions were used to separate CARBO and TROX. C18 and C8 columns with different length and particule sizes were tried to separate both subtances. The best separation was gained by using core-shell pentafluorophenyl propyl column. Firstly a system suitability test was performed to check the sufficiency of the system. Then validation parameters were performed. The limits were determined based on commonly recommended ranges according to EMA Regulations (EMA, 1995). Linearity specificity sensitivity, accuracy, precision parameters were evaluted. Back calculated concentrations of the calibration standards and quality control samples were within ± 15% of the nominal value, except for the LLOQ for which it was within ± 20%. LOD values were also calculated with the following expression:

LOD= 3.33 x SD of the regression line /slope

RESULTS

Different column types and mobile phases were used to separate CARBO and TROX. C18 stationary phase with different

Table 1. System suitability parameters of troxerutin and carbazochrome

Parameter	Value (Troxerutin)	Value (carbazochrome)	Limit (FDA guideline)
Retention Time	5.67	6.07837	-
Peak Width (W)	0.1192	0.1617	-
Tailing (T)	1.32400	1.13889	T ≤ 2
Theoretical Plates (N)	10974	6902	N > 2000
Injection Precision (RSD)	0.424	0.189	RSD% < 1% , n ≥ 5
Resolution (R)	2.9213	2.9213	Rs > 2
Capacity Factor (k)	6.87	7.43	k' > 2

FDA: Center for Drug Evaluation and Research

particle sizes and shapes was tried in order to separate two molecules but the resolution of the chromatograms was not satisfactory. The use of core-shell pentaflorophenyl propyl column provided relevant resolution.

Chromatographic separation of compounds was optimized to provide acceptable resolution, good peak shape and intensity of the response. Mobile phase composition was changed systematically to establish chromatographic conditions giving an acceptable resolution.

System suitability test

System suitability parameters summarized in Table 1 were within acceptable limits (Reviewer Guidance, FDA, 1994). There are also extra data in the table (retention times and peak widths of analytes). Data and calculations were produced by Agilent Chemstation Software.

Specificity

The specificity of method was assessed by analyzing the inactive ingredients of pharmaceutical preparations. Chromatograms of blank samples (sample without drug) and chromatograms of samples were compared for method selectivity (Figure 2). A clear baseline was seen and there was no interference at retention times of analytes.

Limit of quantitation (LOQ) and limit of detection (LOD)

The lower standard 1.00 µg/mL for TROX and 0.05 µg/mL for CARBO on the calibration curve were identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20%. LOD values were also calculated and found to be 0.65 µg/mL and 0.01 µg/mL for TROX and CARBO respectively.

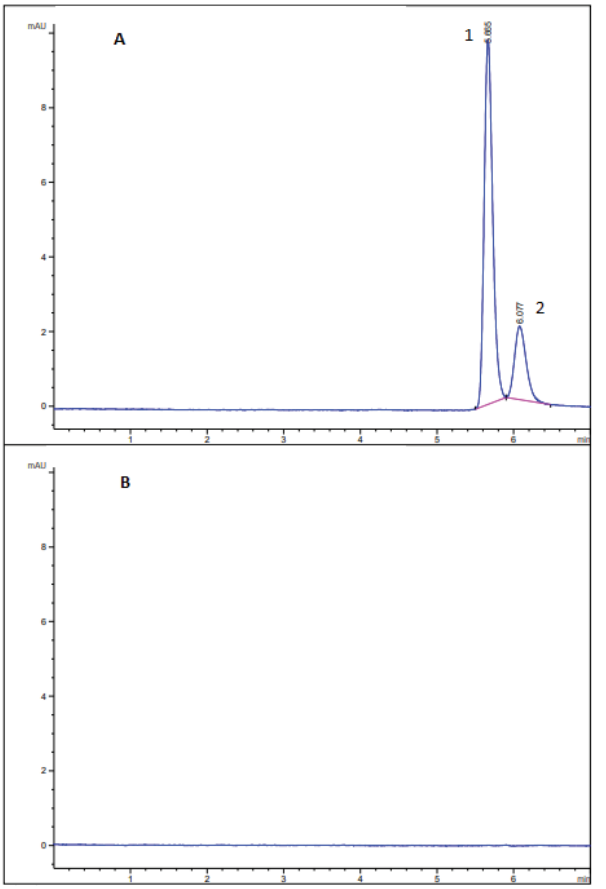

Figure 2. (A) Chromatograms of sample, (B) Placebo. 1: Troxerutin, 2: Carbazochrome.

Linearity

The calibration curves were prepared from five calibration samples within the range of 1.00-20.00 µg/mL including LOQ for TROX and 0.05-1.00 µg/mL including LOQ for CARBO. The standard calibration curves were linear over the concentration with mean r2= 0.9998 for TROX and r2= 0.9999 for CARBO. Regression equation of TROX was (y= 23.37416.x-1.24667) and regression equation of CARBO was (y= 72.58107.x -0.520126).

Accuracy and precision

Repeatability - intermediate precision and accuracy of TROX and CARBO were carried out within the range of calibration curves using six individual quality control samples at four concentrations including low, medium and high concentration QC samples according to EMA guidlines (EMA, 1995). The precision was expressed as coefficient of variation (CV%), accuracy was expressed as relative error (Table 2, Table 3). Analysis of these QC samples were carried out on three separate days.

The values of RD and CV% were within ±15% for the QC samples, except for the LLOQ which was within ±20%. The methods are quite accurate and precise.

Table 2. Results of accuracy and intermediate precision for troxerutin

Sample	Concentration (µg/mL)	Average (µg/mL)	RD	SD	CV%	n
QC1	1.00	1.13	12.58	0.06	5.62	18
QC2	3.00	3.10	3.42	0.08	2.48	18
QC3	15.00	14.82	-1.17	0.27	1.80	14
QC4	20.00	19.90	-0.52	0.46	2.29	18

n: Number of replicate at same concentration

Table 3. Results of accuracy and intermediate precision for carbazochrome

Sample	Concentration (µg/mL)	Average (µg/mL)	RD	SD	CV%	n
QC1	0.05	0.05	-2.06	0.01	11.38	15
QC2	0.30	0.32	6.81	0.02	5.40	18
QC3	0.70	0.72	3.29	0.04	6.08	18
QC4	1.00	1.02	1.65	0.07	7.41	18

n: Number of replicate at same concentration

Recovery of the drug substances

Calculation of the percent recovery was made using equation of calibration curve. They were found to be 101.18±1.13% and 103.43±2.75% for TROX and CARBO respectively.

DISCUSSION

TROX and CARBO have been shown to be safe and effective agents for the treatment of chronic venous insufficiency. There are therefore many separate studies about quantitative analyses of troxerutin and carbazochrome and a few combined studies. Khattab et al. (2015) developed the derivative spectrophotometric method for simultaneous determination of troxerutin and carbazochrome from injectable preparation. The spectrophotometric method is cheap and easy but lack of analysis automation is a major drawback. Abdelrahman et al. (2016) determined carbazochrome and troxerutin from injectable pharmaceuticals using the HPLC and HPTLC-densitometric method. While they used C18 column in their methods to apply to injectable pharmaceuticals, in our study core-shell PFP column was used to separate molecules and the method was applied to tablet forms of combination of troxerutin and carbazochrome. Abdelrahman et al. (2016) provided much better resolution, however the theoretical plates (N) values did not meet the acceptance criteria. We achieved a much better theoretical plates value and a better capacity factor. They did not perform injection precision for system suitability. Tailing factors and linearity values were nearly the same. The developed method was almost five times more sensitive than Abdelrahman et al.'s study. In conclusion, the present method is different and has powerful aspects for the determination of troxerutin and carbazochrome. In this paper, the method based on UV- absorption detection with HPLC technique using core-shell PFP column was developed. The electronic transitions in organic compounds can be determined by UV. TROX and CARBO molecules have conjugated π bonds and free electron pairs which are donated as n. These two compounds show $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The absorption maxima of these two compounds was found to be 348 nm for TROX and 355 nm for CARBO. 350 nm were chosen for determination both of TROX and CARBO.

Sufficient retention and separation of very polar TROX and CARBO were not provided with traditional C18 column. In order to maximize efficiency, chromatographic separations, sensitivity and improve peak capacity, core-shell pentafluorophenyl propyl column was used. Electronegative flourine groups affect polar functional groups of analytes. Also planar interactions of column improve resolution. Interaction between π- π electrons of the carbon ring and π- π electrons of analyte contribute to the increasein retention time in non-acetonitrile mobile phases.

This method exhibited excellent regression in the range of 1.00-20.00 µg/mL including LOQ for TROX including LOQ (r= 0.9998) and 0.05-1.00 µg/mL including LOQ for CARBO (r= 0.9999). The accuracy and precision of the method was given in Table 2 for troxerutin and Table 3 for carbazochrome. RD and CV% terms were used to express the accuracy and precision of the method. The CV% and RD for troxerutin is within the range 1.80 - 5.62 and (-0.52) - 12.58 respectively. The CV% and RD for carbazochrome is within the range 5.40-11.38 and (-2.06)- 6.81 respectively. Values found were evaluated according to EMA guidelines (EMA, 2011). It was seen that they met the acceptance criteria (CV should be maximally 15% (LLOQ: 20%)). Deviation of LOQ samples was higher than the others but they also met the acceptance criteria because of having a wider limit range. In addition, the literature survey shows that most studies with high RSD value have low sensitivity. Hu et al. (2014) developed a method to carbazochrome sodium sulfonate for injection and studied 0.08865 mg/mL-0.4432 mg/mL calibra-

tion range. Hepsebah et al. (2014) determined troxerutin and calcium dobesilate simultaneously and found the linearity of the method over the range 62.5-250 µg/mL for both the drugs.

The method developed is sensitive and selective enough for the determination of TROX and CARBO from pharmaceuticals and also this method can be applied to biological material analysis, for example pharmacokinetic and therapeutic drug monitoring studies.

CONCLUSION

The developed method for the determination of TROX-CARBO from pharmaceutical preparation was found to be accurate, precise, selective, and suitable for the quality control analysis. In this study, we demonstrated the relevant separation with a core shell column technique. Separation with core shell technology column resulted in significant reduction in solvent consumption and time, combined with good resolution for compounds and better selectivity without the need of high cost instruments such as mass spectrometry.

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Original Article

Comparative morphological, fruit anatomical and micromorphological studies of two *Trinia* species

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ABSTRACT

Background and Aims: The aim of the present study was to review and compare the morphological, micromorphological, and anatomical characteristics between *Trinia scabra* Boiss. & Noe and *T. glauca* (L.) Dumort.
Methods: The fruit micromorphologies were examined using scanning electron microscopy. For anatomical studies, the Paraffin method was used. Transverse sections were cut using a rotary microtome and then were stained with safranin.
Results: Expanded descriptions, phenologies, geographic distributions, and photos of the *T. scabra* and *T. glauca* are provided and the differences between the two species are discussed.
The morphological, micromorphological, and anatomical characteristics of *T. glauca* and *T. scabra* are presented. Fruit anatomy is the best discriminating characteristic between the two species, which is entirely different in each. There is one vallecular vitta and there are two commissural vittae in *T. scabra*, but one to three vallecular vittae and one to two commissural vittae in *T. glauca*. The vittae are relatively large in *T. scabra* but small in *T. glauca*. There are no sclerenchyma cells toward the end of the ribs in *T. scabra* although they are found toward the end of the ribs in *T. glauca*. Also, the presence of *T. leiogona* C.A.Mey in Turkey has been reported by Grossgeim in *Flora Kavkaza* and confirmed our study.

Keywords: *Trinia*, morphology, Apiaceae, Turkey

INTRODUCTION

The flowering plant family Apiaceae (Umbelliferae) comprises approximately 450 genera and 3700 species (Pimenov & Leonov, 1993). It is widely distributed in the temperature zones of both northern and southern hemispheres and exhibits great diversity in Central Asia (She et al., 2005). The family consists of 105 genera and around 493 species in Turkey.

Trinia Hoffm. is distributed throughout Europe and southwest Asia and comprises approximately 10 species. The genus was revised by Hedge & Lamond (1972) for *Flora of Turkey and the East Aegean Islands*, in which *T. glauca* (L.) Dumort and *T. scabra* Boiss. & Noë, were accepted as two species. Despite that *Trinia* is a small genus, there is considerable confusion about its taxonomy and nomenclature at inter- and intra-generic levels (Wolff, 1910; Uribe-Echebarría, 1990). The high variability in some of the most important characteristics that define and characterize the species are factors for intrageneric confusion. It is classically accepted that *Trinia* species contain unisexual flowers (dioecious or rarely monoecious plants) (Cannon, 1968), although this characteristic is still being studied, because hermaphrodite flowers have occasionally been observed, which could lead researchers to consider that some species in this genus are polygamous (Ferrer-Gallego et al., 2013; Gómez-Navarro et al., 2015). In addition, the height, branching system, number and length of rays, and length of leaf lobes vary considerably between male and female plants of the same species (Hedge & Lamond, 1972).

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Trinia was established by Hoffm. in 1814 as a genus that is distinct from *Rumia*. *Trinia* and *Rumia* Hoffm. were repeatedly subjected to revisions and many researchers transferred species from one genus to the other or reunited (Fedoronchuk, 1978). In *Flora Altaica* (Ledebour, Meyer, & Bunge, 1829), *R. seseloides*, described by Hoffmann, was transferred into *Trinia*; therefore, *Trinia* and *Rumia* were combined as one genus. In *Prodromus Systematis Naturalis* (De Candolle, 1830), *Trinia* and *Rumia* were classified in tribe Ammineae as separate genera. Later in *Flora Rossica* (1844), Ledebour not only separated these genera, but placed them to different tribes. *Trinia* was placed in tribe Ammineae and *Rumia* was placed tribe Seselineae. Boissier (1872), following De Candolle (1830), evaluated two genera in tribe Ammineae. The results of the study by Ajani et al. (2008) showed that *T. hispida* Hoffm. was very closely related to members of tribe Selineae and Downie et al. (2010) confirmed these positions and considered *Rumia* to be a separate genus.

In the current study, we contribute to the knowledge about *T. scabra* and *T. glauca*, and the presence of *T. leiogona* in Turkey is confirmed. In this paper, the macro- and micromorphological properties of the fruits were investigated. A detailed anatomical description of the cross sections of the mature fruits is provided. An expanded description of the species is also provided.

MATERIALS AND METHODS

Samples of *Trinia scabra* and *T. glauca* were collected from different localities in Turkey (Appendix 1). Plant samples were identified according to Shishkin (1951), Canon (1968), Hedge and Lamond (1972), Rechinger (1987), Güner et al. (2012). The abbreviations of the authors of plant names were checked from Brummitt and Powell (1992).

The fruits were directly mounted on the prepared stubs and coated with gold for SEM studies. Photographs were taken with a Zeiss LS-10 after coating with a Polaron SC7620 sputter coater.

Mature fruits were kept in 70% ethanol. Each mericarp was rehydrated and placed in formalin-acetic acid- alcohol (1:1:8) for a minimum of 24 h. Rehydrated materials were embedded into paraffin blocks following the traditional paraffin section method. A transverse section about 5-10 µm thick was cut using a Thermo microtome and stained with a safranin solution. Micrographs were taken using a Nikon light microscope.

RESULTS AND DISCUSSION

***Trinia scabra* Boiss. & Noe**

Erect biennial with much branched, herbs. Root fusiform, 6-15 mm in diam., covered with densely fibrous collar at the base. Stem 15-35 cm, puberulous in the lower part and sparsely puberulous in the upper part, ribbed, partly glaucous. Basal leaves 2-pinnate, ovate-lanceolate, 4-20 x 2-6 cm, petiole with broadly membranous sheathing base, terminal segments linear-lanceolate, 2-12 mm long, acute to acuminate at apex, margins and veins usually scabrid. Median and upper cauline leaves gradually smaller towards flowering part; terminal segments mostly linear, up to 25 mm long. Umbel compound, rarely simple, 3-8 rayed, 0.3-4.5 (-5) cm long, very unequal, ribbed, 0.5-0.8 mm thick, usually glabrous. Bracts 0-3, unequal, 0.9-7.5 x c. 0.5 mm, simple, rarely divided into linear lobes. Bracteoles 1-3, ± linear, unequal, up to 6 mm. Umbellula (4-)7-15 flowered, 5-10 mm in diam, pedicels 0-6 (-10) mm, unequal on female plants, ± equal on the male. Sepal obsolete, petals white, incurved at apex, c. 0.6 mm long, ovate. Fruit broadly oblong-ovoid, laterally compressed. Mericarp oblong-elliptic, 2.8-3.2 x 1.2- 1.8 mm, scabrid. Stylopodium depressed. Stylus short, recurved, flat (Figure 1 and 2).

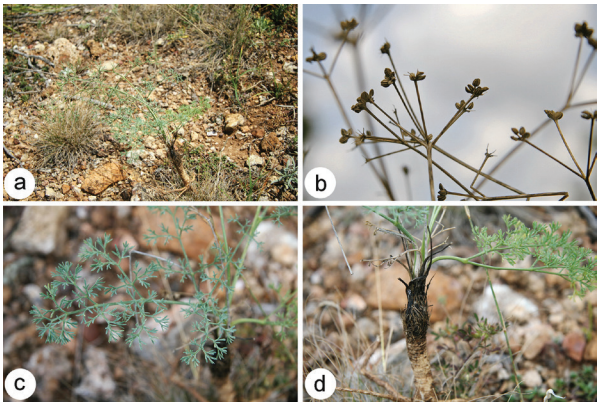

Figure 1. *Trinia scabra* and a- habitus, b- fruit, c- basal leaves, d- root.

Figure 2. *Trinia scabra*. **a- b-** fruit, **c-** stem, **d-** petiolulate, **e-** leaf, **f-** ultimate segment.

Phenology: Flowering time from June to July.

Distribution: This taxon is known only from Anatolia. It is endemic to Turkey.

Phytogeographic region: Irano-Turanian

***Trinia glauca* (L.) Dumort**

Perennial with much-branched, ± glaucous. Root fusiform, 8-15 mm in diam., covered with densely fibrous collar at the base. Stem 15-30(-50) cm, angled, glabrous, ribbed. Lower leaves 2-3-pinnate, petiole with sheating base, terminal segments linear, acute, glabrous or rarely margins and veins scabrid. Median and upper cauline leaves gradually smaller towards the flowering part; terminal segments mostly linear, 3-20(-35) mm. Umbel (3-)4-8(10) rayed, (5-) 8-30 mm long, subequal, ribbed, glabrous. Bracts 0-1, entire or trifid. Bracteoles absent or few, ± linear, up to 1-2 mm. Umbellula 3-8, peduncles1-7 mm, unequal on female plants. Sepal obsolete, petals white, incurved at apex. Fruit elliptic to oblong, glabrous, 2-3(-3.3) x (1-)1.5-2 mm., slightly laterally compressed. Stylopodium short conical, undulate at margin. Stylus short, recurved, 0.5-0.8 mm long (Figure 3 and 4).

Figure 3. *Trinia glauca*. **a-** habitus, **b-** fruit, **c-** basal leaves, **d-** root.

Figure 4. *Trinia glauca*. **a-** umbellule, **b-** fruit, **c-** bracteole, **d-** stem, **e-** leaf, **f-** ultimate segment.

Phenology: Flowering time from June to July.

Distribution: West & South Europe, northwards of S. England.

Micromorphological results

The fruit of *Trinia scabra* is broadly oblong. It is composed of two equal mericarps which easily split open at maturity. Mericarps are oblong-elliptic and 2.8-3.2 x 1.2-1.8 mm, somewhat laterally compressed. Each mericarp has five strongly projecting primary ribs, all ribs nearly equidistant each other, with four deep valleculae. Stylus short, recurved, flat and 0.8-1.2 mm long. The ornamentation of the fruit surface is striate. The pericarp surface is covered with hairs (Figure 5).

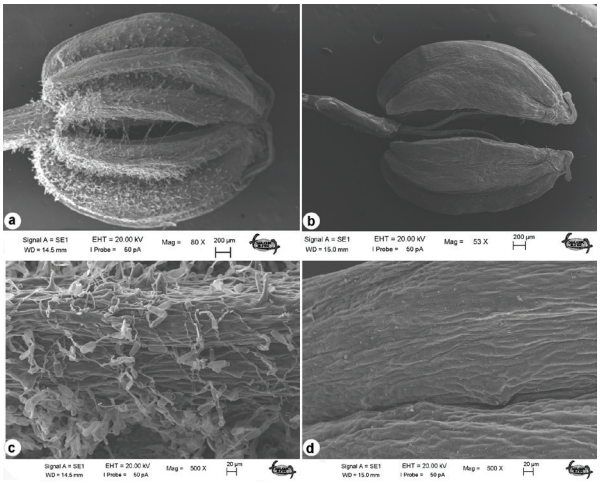

Figure 5. SEM photos of fruits of *Trinia scabra* **a-** general shape of fruit, **c-** surface details, *T. glauca* **b-** general shape of fruit, **d-** surface details.

The fruit of *Trinia glauca* is elliptic to oblong, glabrous, 2-3 x (1-)1.5-2 mm. It is composed of two equal mericarps that easily split open at maturity. The mericarps are ovoid to oblong, and somewhat laterally compressed. Each mericarp has five strongly projecting primary ribs, with the three dorsal ribs wider and closer to each other than the others, and the two marginal ribs somewhat narrower with four deep valleculae. The stylopodium is short conical, and undulate at the margin. The stylus is short (0.5-0.8 mm long), and recurved. The pericarp surface contains elongated rectangular-like or irregularly shaped pentagonal cells. Fine striations can be seen on the cell surfaces (Figure 5).

Anatomical results

The mericarps of *T. scabra* are nearly semi-circular in the transverse section. They have five strongly projecting ribs. Secondary ribs are absent. The ribs are 0.4-0.6 mm long in depth. The width of furrows are 0.15-0.30 mm, they slightly narrow at the base and top of the fruit. The exocarp is a single layer that consists of rectangular-like cells (10–30 x 5–12 µm) with a thickened outer wall covered with a thick, scaly, or nearly smooth cuticle layer. The exocarp layer is interrupted at the base of the marginal ribs from the commissural side. The mericarp consists of multi-rowed parenchymatous and sclerenchymatous cells. The rib ducts within the ribs are surrounded by mericarp cells and a vascular bundle. They are round, 120–250 µm in diameter, and occupy nearly the entire rib cavity. The vittae are relatively large (150–200 x 60–100 µm), lined with thin-walled epithelial cells, and some what destroyed in the mature fruit. There is one vallecular vitta and there are two commissural vit-

tae. There are two vascular bundles on both sides of the base of the rib duct. The endocarp is 20–30 µm thick and composed of two to three layers of thin-walled cells that are heavily elongated in a longitudinal direction. The seed coat adheres to the inner part of the pericarp. The endosperm on the transverse section is oval and slightly impressed into the commissural side. Endosperm cells are large and filled with dense granular contents (Figure 6).

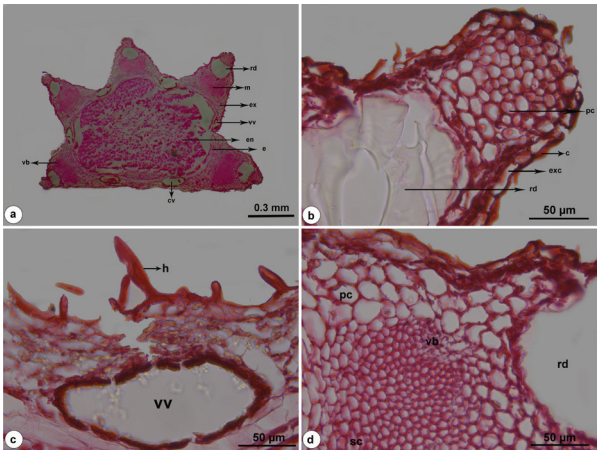

Figure 6. Fruit anatomy of *Trinia scabra* **a-** mericarp; **b-** dorsal rib area; **c-** vallecular view; **d-** the view of the vascular bundle and rib duct. (rd-rib duct, m-mesocarp, e-endocarp, ex-exocarp, cv-commissural vitta, vv-vallecular vitta, vb-vascular bundles, en-endosperm, pc-parenchymatous cells, sc-sclerenchyma cells, exc-exocarpcell, c-cuticle, h-hair, s-seed coat, enc-endosperm cell with glandular content).

In the transverse section, mericarps of *T. glauca* are nearly semi-circular. They have five distinctly angled and strongly projecting ribs. Secondary ribs are very rare. Ribs are 0.1- 0.2 mm long in depth. The furrows are wide, 0.18-0.40 mm, they narrow at the base and top of the fruit. The exocarp is a single layer. It consists of rectangular-like cells (5-20x5-12 µm) with a strongly thickened outer wall covered with a thick, weakly scaly or almost smooth cuticle layer. At the base of the marginal ribs from the commissural side, the exocarp layer is interrupted. The mericarp consists of multi-rowed parenchymatous and sclerenchymatous cells. Rib ducts are located into the ribs and surrounded by mericarp cells and vascular bundle. They are large, elliptic or round (150-320 x 70-260 µm) and occupy almost the entire cavity of the rib. Mesocarp tissue under the rib ducts is formed 4-7 layer sclerenchyma cells, which at the base of the ribs gradually turns into oblong cells of a larger diameter with thickened walls. Also, there are several layer sclerenchyma cells towards the end of ribs. Vittae are small, lined with thin-walled epithelial cells, often destroyed in a mature fruit. The number of the vallecular vittae is 1-3 and the number of the commissural vittae is 1-2. There are two vascular bundles which are collateral type located on both sides of the base of the rib duct. Endocarp is 30-45 µm in thickness. It is composed of 2 -3 layers of thin-walled cells, strongly elongated in the longitudinal direction. The seed coat is closely adherent to the inner part of the pericarp and is represented by an external epidermal layer well preserved in the mature fruit, the cells of which are thin-walled, strongly elongated in the longitudinal direction. Between this layer and the endosperm there is a thin layer of lysed cells. Endosperm on the transverse section has an oval shape, slightly impressed in the comissural side. Endosperm cells are large, densely filled with granular contents (Figure 7).

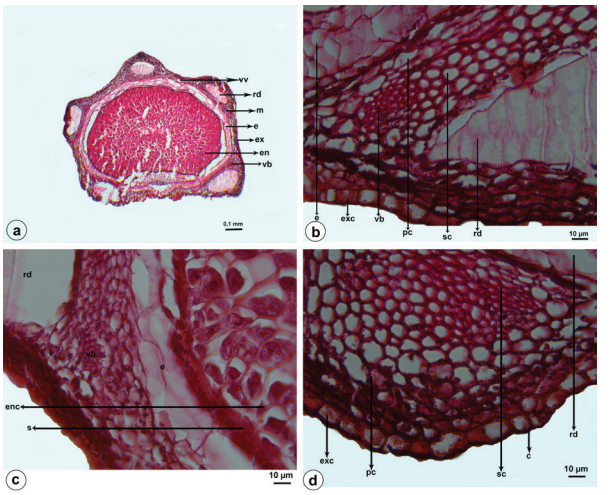

Figure 7. Fruit anatomy of *Trinia glauca* **a-** mericarp; **b-** pericarp view; **c-** marginal view of mericarp; **d-** dorsal rib area.

DISCUSSION

Trinia comprises approximately 10 species that are distributed throughout Europe and southwest Asia. In *Flora of the U.S.S.R.*, eight *Trinia* species were divided into two sections—*Leptopus* and *Pachypus*—based on pedicel thickness (Shishkin, 1951).

The genus was revised by Hedge & Lamond (1972) for Flora of Turkey and the East Aegean Islands, in which *T. glauca* (L.) Dumort and *T. scabra* Boiss. &Noë, were accepted as two species. In Flora Kavkaza (1967), Grossheim noted that *T. leiogona* was collected from two different localities (A8 Erzurum and A9 Kars) in Turkey. The presence of the species in Turkey is confirmed here.

Fruit and inflorescence characteristics are considered to be the most important features for identifying *Trinia* species. The presence or absence of bracteoles, glabrous or scabrous fruit, rib shape, and the pedicel thickness in the fruit are important diagnostic features and are used to determine species limits.

Fedoronchuk (1978) divided the genus *Trinia* into 2 subgenera (subgenus *Trinia* and subgenus *Triniella*) based on primary and secondary ribs, leaf petiole anatomy, micromorphology of pollen grains, chromosome numbers. Subgenus *Triniella* included two species,—*T. daleshampii* (Ten.) Janchen and *T. guicciardii* (Boiss. &Heldr.) Drude. Subgenus *Trinia* was divided into three sections—*Pachypus*, *Trinia*, and *Levigatus*.

Trinia glauca is distributed from Europe to western Asia. The northwestern and western parts of Turkey and Crimea form the species' eastern distribution boundary. *Trinia glauca* is known only from the Karabük, Bursa, and Kırklareli regions of Turkey.

According to Hedge & Lamond (1972), *T. glauca* is distinguished from *T. scabra* by its one to three bracteoles and scabrid leaf veins and margins; however, it appears that these characteristics might not be suitable for distinguishing these species. The presence of bracteoles in *T. glauca* was mentioned by Jury (2003) and Aizpuru et al. (1999) has mentioned that its leaf margin is scabrid. Fruit anatomy is the best discriminating characteristic between the two species, which is entirely different in each. There is one vallecular vitta and there are two commissural vittae in *T. scabra*, but one to three vallecular vittae and one to two commissural vittae in *T. glauca*. The vittae are relatively large in *T. scabra* but small in *T. glauca*. There are no sclerenchyma cells toward the end of the ribs in *T. scabra*although they are found toward the end of the ribs in *T. glauca*.

Trinia scabra was described by Boissier & Noë (1856), who considered *T. scabra*and *T. leiogona* to be two separate species. (Boissier, 1856). Since its description (Boissier, 1856), a detailed study on it had never been conducted. Moreover, the description in the protologue is short and contains no information about fruit; therefore, species delimitations are very difficult or even impossible. In *Flora Orientalis*, the fruit of *T. scabra*was described as "fructus juniorisasperuli tandem glabriovatijugiscrassiselevatisobtusis, valleculis late univittatis". The stem was described "pruinoso-scabra" in both the protologue and *Flora Orientalis*. Hedge & Lamond (1972) provided an expanded description of *T. scabra* and noted that the fruit of the species is glabrous or scabrid, and the stem is puberulous or glabrous. In this description, *T. leiogona* and *T. scabra* were evaluated as the same species, therefore, this description is artificial.

Trinia leiogona has a limited geographic distribution that comprises Iran, Armenia, Azerbaijan, south Russia, and northeast Turkey. The west shore of the Caspian Sea is the northern distribution boundary and south Russia and northeast Turkey comprise the western distribution boundary of this species.

Table 1. Comparison of the morphological features of the *Trinia glauca, T. hispida, T. leiogona* and *T. scabra*

Species Characters	*Triniahispida*	*T. leiogona*	*T. scabra*	*T. glauca*
Stem	much-branched, up to 35 cm	single, 10-50 cm high	much branched, 15-35 cm	much branched, 15-25(-50) cm.
Stem hairy	glabrous to hispid with short stiff hairs hairy or sub-glabrous	very short scabrous-hairy or glabrous	densely puberulous in the lower part and sparsely puberulous in the upper part	glabrous
Lower leaves	10-20 cm long, bi- or tripinnate	8-12 x 4-6 cm, ovato-oblonga,tripinnate	4-20 x 2-6 cm, ovate-lanceolate, 2-pinnate	2- to 3-pinnate
Umbel	up to 10 rayed (male plants) and up to 9 markedly unequally (female plants)	unequal, 5-7 rayed	very unequal, 3-11 rayed	subequal, 4-8(10) rayed
Bracts and bracteole	bracteole absent	bracts and bracteole 0-1	bracts 0-3, bracteoles 1-3	bracts 0-1, trifid or simple, bracteole 0-5
Umbellula	9-20	7-11 , 2-19 mm long	(4) 7-18	3-8, (5-) 8-30 mm long
Fruit size	3-4 x 2.5-3 mm	3,5-4x 2-2.5 mm	2.8-3.2 x 1.2- 1.8 mm	2-3,3 x (1-)1.5-2 mm
Fruit shape	broadly ovoid	ovoid, ovoid-oblong	broadly oblong	elliptic to oblong
Fruit hairs	nearly always covered with short stiff hairs	glabrous	scabrid	glabrous
Peduncle	unequal, (1-2)- 4 mm long	very unequal, 1-19 mm long, thickened in fruit	unequal (female plants), ± equal (male plants), 0-10 (-15) mm,	unequal on female plants, ± equal (male plants), 1-7 mm
Fruit surface ornamentation	-	-	striate	contains elongated rectangular-like and irregularly shaped pentagonal cells. Fine striations can be seen on the cell surfaces
Stylus length	-	-	0.8-1.2 mm long	0.5–0.8 mm long
Secondary ribs	-	-	absent	very rarely
Vallecular vitta	-	-	large, one vallecular vitta	small often destroyed in a mature fruit 1-3 vallecular vittae
Commissural vittae	-	-	two commissural vittae	1-2 commissural vittae

According to Fedoronchuk (1979) and Shner, Pimenov, & Kljuykov (in Kamari, Blanche, & Garbari 2004) *T. scabra* and *T. leiogona* are synonyms, the latter name having priority in species ranking; however, Hedge & Lamond (1972) noted that the nomenclature of *T. scabra* should be investigated together with that of the Caucasian *T. leiogona*, which differs from *T. scabra* by its absence of bracteoles and indumentum. The presence of scabrid hairs on the fruit of *T. scabra* suggests that the species is more closely related to *T. hispida* than to *T. leiogona*. According to the regional flora, the presence of bracteoles in *T. scabra* separate the species from *T. hispida* and *T. leiogona*. According to the description in *Flora of U.S.S.R.*, both the bracts and bracteoles in *T. hispida* are absent, however, according to the description in *European Flora*, only the bracteoles are absent. Bracteoles are also absent in *T. leiogona* according to descriptions in both publications. When we examined images of *T. leiogona* from the Moscow Herbarium (MW0698699) and the specimens collected from Turkey, we noted that some specimens of *T. leiogona* had both bracts and bracteoles. The fruits of *T. scabra*, *T. leiogona*, and *T. hispida* also have a similar anatomicalstructure, making them unsuitable characteristics for separating this complex. Determination of intraspecific variations of *T. hispida* and *T. leiogona* is a critical consideration for defining a synonym for these species. A comparison of the morphological features of *T. glauca*, *T. hispida*, *T. leiogona*, and *T. scabra* is presented in Table 1.

The morphological, micromorphological, and anatomical results from this study clearly show that enough differences exist between *T. glauca* and *T. scabra* and support their treatment as distinct species. Our findings indicate that *T. scabra* differs from *T. leiogona,* therefore, this should be accepted at species ranking. Nonetheless, further studies, including compilations of morphological and micromorphological data, as well as new sequences of taxa related to *Trinia*, such as *T. hispida* and *T. leiogona*, will shed new light on refining the taxonomic relationships among all these species of concern.

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APPENDIX 1. Examined species

Trinia glauca

A4 Karabük: Keltepe, 1900 m, 11.09.1997, steppe, N.A. 3058 & A. Kaya (GAZI); Karaağaç, Keltepe, 1855 m., 21.07.2018, stony steppe, 41°05'20"N, 32°27'40"E, M.Çelik 533.

Spain: Valladolid: Tiedra, 08.06.1981 (MA 310705); Valladolid: Encinas de Esgueva monte de encinas, 12.06.1981, F. Alonso 478JF (MA 424183); Valladolid:: Canillas de Esgueva, 22.06.1984 (MA 311202); Valensia S de Enguera, XJ81, 900 m, mattorales calciolas, 12.06.1984, G. Mateo, R. Figuerola sn. (MA 426567); Leon: Puente Orugo, 30T TN5060, en grietas de roca caliza, 01.07.1983, E. Bayon, S. Castroviejo 8594, P. Galan & G. Nieto (MA 426305).

Trinia scabra

B3 Eskişehir: İnönü, from İnönü to Kütahya, 8. km, 990 m, 02.07.2015, rocky slopes and open *Quercus*, 39°47'45"N, 30°12'56"E, M.Çelik 379; ibid. 22.07.2018, M.Çelik 534 & Ö.Çetin.

Trinia leiogona

B5 Nevşehir: Zelve, Akdağ, 1280-1300 m, 19.06.1989, *Thymus* steppe, M.Vural 5233 & et al.

A9 Kars: Arpaçay, from Doğruyol to Çanaksu, c. 1 km, 2008 m., 04.08.2018, rocky steppe, 41°03'25.81"K- 43°18'59.74"D, M.Çelik 535.

A9 Kars: Arpaçay, Karakale, c. 2 km north of the Karakale, 2048 m., 04.08.2018, rocky steppe, 40°53'13.94"K- 43°27'13.44"D, M.Çelik 536.

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Original Article

In vitro investigation of *Sorbus domestica* as an enzyme inhibitor

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ABSTRACT

Background and Aims: Finding new therapeutic enzyme inhibitors by investigating especially medicinal plants is an important research area. The fruits and leaves of *Sorbus domestica* (service tree) are used as food and folk remedies due to astringent, antidiabetic, diuretic, antiinflammatory, antiatherogenic, antidiarrhoeal, vasoprotective, and vasorelaxant activities, and also used commercially as a vitamin and antioxidant. In this study, the therapeutic effect of *S. domestica* against diabetes, Alzheimer's disease, aging, and hyperuricemia was investigated.
Methods: α-Glucosidase, α-amylase, acetylcholinesterase (AChE), butyrylcholinesterase (BChE), elastase and xanthine oxidase (XO) inhibitory activities of the fruit extract from *S. domestica* were measured.
Results: The extract showed inhibitory activity against α-glucosidase, α-amylase, BChE, elastase, and XO whereas AChE inhibitory activity of the extract could not be determined. Moreover, the inhibition effects of the extract against α-glucosidase and elastase were more effective than the standard drugs acarbose and ursolic acid, respectively.
Conclusion: *S. domestica* can be evaluated as a potential source for a new therapeutic agent.

Keywords: α-glucosidase, α-amylase, acetylcholinesterase, butyrylcholinesterase, elastase, xanthine oxidase

INTRODUCTION

The discovery of enzyme inhibitors is an active area of research in biochemistry and pharmacology. There are many drugs that act as reversible/irreversible enzyme inhibitors (Balbaa & Ashry, 2012). α-Amylase (EC 3.2.1.1) and α-glucosidase (EC 3.2.1.20) are carbohydrate-processing enzymes present in the gastrointestinal tract. Postprandial hyperglycemia could be decreased through the inhibition of these enzymes. Therapeutic inhibitors of α-glucosidase such as acarbose have been used for the management of hyperglycemia in diabetes patients (Oboh, Ogunsuyi, Ogunbadejo, & Adefegha, 2016). Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are enzymes that catalyze the hydrolysis of acetylcholine into choline and acetic acid, an essential process for the regeneration of the cholinergic neurotransmission. Cholinesterase inhibitors increase the synaptic level of the neurotransmitter and therefore are used in the symptomatic treatment of Alzheimer's disease, which is the most common reason for dementia (Gülçin et al., 2016; Türkan, Huyut, Taslimi, & Gülçin, 2019). Elastase (EC 3.4.21.36) is a member of the proteases, which is responsible for the breakdown of elastin as well as collagen, fibronectin, and other extracellular matrix proteins providing elasticity to connective tissues (Azmi, Hashim, Hashim, Halimoon, & Majid, 2014; Shukla, Park, Park, Lee, & Kim, 2017). The inhibitors of elastase have the potential to be cosmetic ingredients in combating skin aging due to their usefulness in preventing the loss of skin elasticity and sagging (Azmi et al., 2014). Xanthine oxidase (XO; EC 1.2.3.2) catalyzes the oxidation

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of hypoxanthine, and this reaction is terminated by the formation of uric acid. The overproduction or underexcretion of uric acid leads to hyperuricemia ending as gout. The use of XO inhibitors is one of the medications to cure gout by blocking the formation of uric acid (Harrad & Amine, 2016).

Plants are perfect sources to find new inhibitor compounds for the treatment of diseases (Balbaa & Ashry, 2012). *Sorbus* genus (Rosaceae) comprises more than 250 species and spread throughout the various parts of the World (Olszewska & Michel, 2009). *S. domestica* is a tree that opens white flowers in May-June and usually 5-10 m in length (Baytop, 1999). The fruit of *S. domestica* is a pome 2-3.5 cm long and has greenish-yellow color (Brus, Ballian, Bogunić, Bobinac, & IdžOjtić, 2011). The leaves and fruits of *S. domestica* are used in traditional medicine for the treatment of various diseases due to their astringent, antidiarrheal, diuretic, anti-inflammatory, anti-atherogenic, vasoprotective, vasorelaxant, and antidiabetic effects, as well as used in food industry (as antioxidant agents in beverages production and sweet/jam production) (Baytop, 1999; Ölschläger, Milde, Schempp, & Treutter, 2004; Termentzi, Kefalas, & Kokkalou, 2006; Kültür, 2007; Olszewska & Michel, 2009). In Turkey, the edible fruits are called "üvez" and "börtlücen" (Kültür, 2007). Previous studies have demonstrated the inhibitory potential of some *Sorbus* species (*S. torminalis* and *S. aucuparia*) on different enzymes (McDougall, Kulkarni, & Stewart, 2009; Boath, Stewart, & McDougall, 2012; Ivanov, Garbuz, Malfanov, & Ptitsyn, 2013; Hasbal, Yilmaz-Ozden, & Can, 2015; Hasbal, Yilmaz-Ozden, & Can, 2017; Olszewska et al., 2019). Also, limited studies have shown that *S. domestica* inhibits the aldose reductase, lipoxygenase, and hyaluronidase (Termentzi, Kefalas, & Kokkalou, 2008; Matczak et al., 2018). In this study, the inhibitory activity of the water extract from *S. domestica* fruits on α-glucosidase, α-amylase, AChE, BChE, elastase, and XO was examined for the first time.

MATERIAL AND METHODS

Chemicals

3,5-dinitrosalicylic acid (DNS), 5,5'-dithiobis(2-nitrobenzoicacid) (DTNB), α-amylase, α-glucosidase, acarbose, acetylthiocholine iodide (ATChI), AChE, BChE, butyrylthiocholine iodide (BTChI), elastase from porcine pancreas, galantamine hydrobromide, *p*-nitrophenyl α-D-glucopyranoside (*p*NPG), *N*-succinyl-Ala-Ala-Ala-p-nitroanilide (STANA), starch, xanthine, and xanthine oxidase (XO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were analytical grade.

Preparation of the plant extract

Fruits of *S. domestica* were collected from Bartın in the Black Sea region of Turkey (ISTE 95595). The fruits were air-dried and cut into small pieces. The extraction was performed by the decoction method. Fifteen g of the fruits were refluxed with distilled water for 3 hours. The extract was filtered and the solvent was evaporated (Buchi, Switzerland) to dryness under reduced pressure, then stored in -20°C until needed. For the biochemical assays, the extract was dissolved in distilled water.

α-Glucosidase inhibitory activity

The α-glucosidase inhibitory effect of the extract was investigated according to the procedure of Bothon et al., (2013). Twenty-five µL of the fruit extract were mixed with 75 µL of sodium phosphate buffer (0.1 M; pH 6.8). Then, 50 µL of α-glucosidase solution (1 U/mL) were added, and the mixture was preincubated at 37°C for 10 minutes. Thereafter, 50 µL of *p*NPG solution (5 mM) were added, and the absorbance change was measured at 405 nm. Acarbose was used as a standard, and the control was prepared without inhibitor. The percent inhibition of the enzyme was calculated according to the following formula:

$$Inhibition\ level\ (\%) = \left(1 - \frac{Reaction\ rate\ of\ sample\ at\ 405\ nm}{Reaction\ rate\ of\ control\ at\ 405\ nm}\right) \times 100$$

α-Amylase inhibitory activity

The inhibition of α-amylase was assayed using the DNS method (Ali, Houghton, & Soumyanath, 2006). Ten µL of the fruit extract were incubated with 50 µL of α-amylase solution (3 U/mL) and 40 µL of sodium phosphate buffer (0.1 M; pH 6.8) at 25°C for 10 minutes before adding the substrate solution. The reaction was initiated by adding 50 µL of starch solution (0.75%). After 5 minutes, the reaction was stopped by adding 75 µL of DNS color reagent (96 mM DNS and 5.31 M potassium sodium tartarate in 2 M NaOH). The mixtures were heated at 85°C for 15 minutes. After cooling, the mixture was diluted 4-fold with distilled water, and absorbance was measured at 540 nm. Acarbose was used as a standard and the control was prepared without an inhibitor. The percentage inhibition of the enzyme was calculated by the following equation:

$$Inhibition\ level\ (\%) = \left(1 - \frac{Absorbance\ of\ sample\ at\ 540\ nm}{Absorbance\ of\ control\ at\ 540\ nm}\right) \times 100$$

Cholinesterase inhibitory activity

The inhibitory activities of the extract on AChE and BChE were determined using the method of Ellman, Courtney, Andres, & Featherstone, (1961) with slight modifications. Twenty µL of the extract and 220 µL of Ellman solution (318 mM DTNB, and 955 mM ATChI/BTChI in phosphate buffer; pH 7.5) were mixed, then 10 µL of AChE/BChE solution (0.5 U/mL) were added, and the absorbance change was monitored at 412 nm. Galantamine was used as a standard and the control was prepared without an inhibitor. The percent inhibition of the AChE/BChE was calculated by the following formula:

$$Inhibition\ level\ (\%) = \left(1 - \frac{Reaction\ rate\ of\ sample\ at\ 412\ nm}{Reaction\ rate\ of\ control\ at\ 412\ nm}\right) \times 100$$

Elastase inhibitory activity

The elastase inhibitory activity of the extract was determined according to the method of Moon, Yim, Song, Lee, & Hyun, (2010) with slight modifications. Fifty µL of the extract were preincubated with 50 µL of elastase solution (0.16 U/mL) and 900 µL of Tris-HCl buffer (0.2 M; pH 7.8) at 37°C for 15 minutes before adding the substrate solution. Then, 50 µL of STANA solution (5 mM) were added, and the mixture was incubated at 37°C for 30 minutes. The release of *p*-nitroaniline was measured at 410 nm. Ursolic acid was used as a standard and the control was prepared without an inhibitor. The percentage inhibition of elastase was calculated by the following formula:

$$Inhibition\ level\ (\%) = \left(1 - \frac{Absorbance\ of\ sample\ at\ 410\ nm}{Absorbance\ of\ control\ at\ 410\ nm}\right) \times 100$$

XO inhibitory activity

XO inhibitory activity of the extract was assayed by the method of Kalckar & Shafran (1947) with slight modifications. One mL of the extract was preincubated with 0.1 mL of XO solution (0.04 U/mL) and 2.9 mL of sodium phosphate buffer (50 mM; pH 7.5) at 25°C for 15 minutes before adding the substrate solution. Then, 2 mL of xanthine solution (150 mM) were added, and the mixture was incubated at 25°C for 30 minutes. The reaction was stopped by adding 1 mL of HCl solution (1 N), and the absorbance was measured at 290 nm. Allopurinol was used as a standard and the control was prepared without an inhibitor. The percentage inhibition of the enzyme calculated with the equation:

$$Inhibition\ level\ (\%) = \left(1 - \frac{Absorbance\ of\ sample\ at\ 290\ nm}{Absorbance\ of\ control\ at\ 290\ nm}\right) \times 100$$

Statistical analysis

All samples were analyzed in triplicate. Results are given as means ± standard deviation (SD). The results were evaluated using unpaired t-test with NCSS statistical computer package and differences were considered significant at $p<0.05$. The half-maximal inhibitory concentration (IC_{50}) values were calculated from dose-response curves using Microsoft Excel.

RESULTS AND DISCUSSION

Medicinal plants used in folk medicine are currently being investigated for pharmaceutical, food and nutraceutical preparations (Fotakis et al., 2016). In the present study, we have investigated the inhibitory activity of *S. domestica* extract on α-glucosidase, α-amylase, AChE, BChE, elastase and XO activities in order to find a new compound as an enzyme inhibitor.

Previous research suggests that fruit and vegetable-rich diets are associated with a reduced incidence of type 2 diabetes. Also, the leaves and fruits of *S. domestica* are traditionally used against diabetes in many countries as well as in Turkey (Baytop, 1999; Termentzi et al., 2006; Kültür, 2007). The results showed that the extract of *S. domestica* exhibited a strong α-glucosidase and weak α-amylase inhibitory property compared with acarbose (Table 1). The results obtained from this study coincide with the traditional use of *S. domestica* in diabetes. α-Glucosidase and α-amylase inhibitory activities of some *Sorbus* species have also been demonstrated in limited studies (Boath et al., 2012; Hasbal et al., 2017; Broholm, Gramsbergen, Nyberg, Jäger, & Staerk, 2019).

Enzyme inhibitory approaches are recognized as one of the most efficient strategies for Alzheimer's disease (Balkan et al., 2018). The Food and Drug Administration approved cholinesterase inhibitors, tacrine, rivastigmine, galantamine, and donepezil as the main drugs for the therapy of early and moderate stages of Alzheimer's disease (Vafadarnejad et al., 2018). *S. domestica* water extract showed a weak ability to inhibit BChE whereas the extract did not show AChE inhibitory activity (Table 1). However, the anti-AChE activity of *S. aucuparia* and *S. torminalis* have been reported previously (Hasbal et al., 2015; Mrkonjć et al., 2017; Ozsoy, Yilmaz-Ozden, Serbetci, Kultur, & Akalin, 2017).

Degradative enzymes such as elastase, responsible for the structural changes in the skin are the target of the novel strategies to delay the symptoms of aging (Boran, 2018). One of the most important functions of elastase in combination with matrix metalloproteinases is to provide tissue repair under normal conditions after the wounding process (Azmi et al., 2014). The inhibitory activity of *S. domestica* water extract on elastase is shown in Table 1. The results revealed that the water extract possesses a remarkable inhibitory activity on elastase compared with ursolic acid. In the literature survey, no previous report has been found about elastase inhibitory activities of the *Sorbus* species.

XO inhibitors are used for the treatment of conditions associated with hyperuricemia such as gout (Wang, Zhang, Pan, & Gong, 2015). In this study, the water extract of *S. domestica* exhibited inhibitory activity against XO similar to allopurinol. On the other hand, Olszewska et al., (2019) reported that flower extracts of *S. aucuparia* showed no inhibitory effects towards XO.

CONCLUSION

In this study, α-glucosidase, α-amylase, AChE, BChE, elastase and XO inhibitory activities of *S. domestica* fruits were evaluated for the first time. Our results showed that the water extract from *S. domestica* showed strong inhibitory activity against α-glucosidase and elastase, suggesting that *S. domestica* may be a potential source of natural compounds for the treatment of type 2 diabetes and skin disorders.

Table 1. α-Glucosidase, α-amylase, AChE, BChE, elastase and XO inhibitory activities of *S. domestica* water extract and respective standards

	Inhibitory activity (IC_{50} mg/mL)					
	α-Glucosidase	**α-Amylase**	**AChE**	**BChE**	**Elastase**	**XO**
S. domestica	0.417±0.024*	8.768±0.247*	ND	26.907±1.605*	0.100±0.001*	0.007±0.001*
Acarbose	0.548±0.021	0.120±0.023				
Galantamine			0.008±0.001	0.094±0.003		
Ursolic acid					0.131±0.009	
Allopurinol						0.001±0.001

Data are presented as the mean of three replicates ± standard deviation. *Significant difference $p<0.05$ versus the respective control substances. IC_{50}; The inhibitory concentration of the extract or standards required to inhibit the activity of the enzyme by 50%. IC_{50} values were calculated from dose-response curves using Microsoft Excel. AChE; Acetylcholinesterase, BChE; butyrylcholinesterase, XO; xanthine oxidase.

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Original Article

LC-MS/MS analyses of *Ziziphora clinopodioides* Lam. from Turkey: Antioxidant, anticholinesterase, antimicrobial and, anticancer activities

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ABSTRACT

Background and Aims: *Ziziphora clinopodioides* is one of the wild edible species used in Turkish folk medicine. The study was aimed to investigate the phenolic compounds of the extracts as well as the antioxidant, enzyme inhibitory, antimicrobial activities, and anticancer potential.
Methods: LC-MS/MS was used to determine phenolic compounds; antioxidant potential was evaluated by using radical scavenging assays; enzyme inhibition assays were used for anticholinesterase, tyrosinase, and urease activities; antimicrobial activities were performed by microdilution method; and XTT bioassay was used for testing anticancer potential.
Results: The LC-MS/MS results indicate that quinic acid (9020.51±73.97 µg/g; 14721.04±120.71 µg/g, respectively) is the major compound, malic acid (1972.95±22.29 µg/g; 2179.04±24.62 µg/g, respectively) and rhoifolin (1044.74±98.31 µg/g; 3593.31±338.13µg/g, respectively) are the abundant compounds in aerial and root extracts. The antioxidant activity results showed that the aerial parts extract has stronger ABTS cation radical and DPPH free radical scavenging activity than the root extract with 40.90±0.19 µg/mL, and 94.27±0.64 µg/mL IC_{50} values, respectively. The extracts showed moderate cupric reducing activity with 1.74 absorbance value at 100 µg/mL. Only the aerial parts extract exhibited weak tyrosinase inhibition (8.60±0.87%) compared with kojic acid (95.26±0.23%) at 200 µg/mL. No activity was observed in urease and anticholinesterase enzymes. Both extracts exhibited moderate antifungal activity against *Candida tropicalis* with 39.06 µg/mL MIC values. The extracts didn't show cytotoxic activity against Renal (A498, UO-31) and Colo (COLO205, KM12) cell lines, also there is no metastatic potential of the extracts against osteosarcoma cell lines (MG63.3, MG63).
Conclusion: *Z. clinopodioides* has rich phytochemical constituents with powerful health benefits, and doesn't have any harmful effect on the body.

Keywords: *Ziziphora clinopodioides*, antioxidant, enzyme inhibition, antimicrobial, anticancer, LC-MS/MS

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INTRODUCTION

Traditionally used wild edible plants have always been a main research interest in the search for new natural products as therapeutic agents. The ethno-directed research is very important and useful to demonstrate that many wild edible plants are nutritionally rich and have potential for medical treatment (Ali-Shtayeh et al., 2008; Heinrich & Gibbons, 2001).

The genus *Ziziphora* (Lamiaceae) is represented by nearly 17 species in the world, and 5 of them (*Z. clinopodioides, Z. capitata* L., *Z. persica* Bunge, *Z. tenuior* L., *Z. taurica* M.Bieb.) are growing in the Mediterranean region, as well as, central, western, and eastern Anatolian regions in Turkey. *Ziziphora* species are strongly aromatic plants, and are called "dağ reyhanı", "nane ruhu", and "filiskin otu" in Turkish (Kaya & Dirmenci, 2012; Selvi, Satil, Martin, Celenk, & Dirmenci, 2015).

Z. clinopodioides is one of the important wild edible plants which has ethnopharmacological uses in traditional Turkish folk medicine. The aerial parts of *Z. clinopodioides* have traditionally been used as stomachic, carminative, and antimicrobial in Anatolia (Gursoy, Sihoglu-Tepe, & Tepe, 2009; Selvi et al., 2015). Incidentally, in the Eastern part of Turkey, it is an ingredient of a very famous special cheese called "herby cheese" with some other aromatic herbs as well (Ozturk & Ercisli, 2007).

A number of investigations have already been conducted on the chemical compositions and biological activities of the essential oils obtained from *Z. clinopodioides* collected from different parts of the World. Accordingly, the major component is pulegone and other main components are thymol, limonene, menthol, isomenthone, 1,8 cineole, piperitenone, carvacrol and *β*–pinene in the essential oil. The differences in the chemical compositions of the essential oils are due to some factors such as geographical locations and climate conditions (Alp et al., 2016; Ghanbarian, Jafari, & Bahmanzadegan, 2017; Khodaverdi-Samani, Pirbalouti, Shirmardi, & Malekpoor, 2015; Maral, Taghikhani, Kaya, & Kırıcı, 2015; Nickavar & Tavakoli, 2016; Okut, Selcuk, Yagmur, & Yildirim, 2018).

Antimicrobial (Celik, Tutar, Karaman, Hepokur, & Atas, 2016; Hamedi, Kargozari, Shotorbani, Mogadam, & Fahimdanesh, 2017; Yasser Shahbazi, 2015), antifungal (Ma et al., 2016), antibacterial (Pakdel et al., 2017; Y Shahbazi, Shavisi, & Mohebi, 2017), antibiofilm (Celik et al., 2016), antioxidant (Alp et al., 2016; Hamedi et al., 2017), anti-inflammatory (Abu-Darwish et al., 2016), and insecticidal (Kheirkhah, Ghasemi, Yazdi, & Rahban, 2015) activities of essential oils obtained from *Ziziphora* species have been reported in recent years.

The flavonoid components of *Z. clinopodioides* extract were identified by using UPLC-Q-TOF-MS. The researchers reported that ten flavonoid compounds were present in the extract: "baicalein, quercetin, hyperoside, quercetin-3-*O*-*β*-*D*-glucopyranoside, apigenin, kaempferol, chrysin, diosimin, linarin and rutin" (Zhang, An, Guo, Yang, & Zhang, 2018).

Many studies exist on the essential oil composition of *Z. clinopodioides* growing in different parts of Turkey. This study focused on the chemical composition and biological activities of the ethanol extracts of *Z. clinopodioides* aerial and root parts collected from Ardahan, Turkey. According to the literature survey, there has never been a study conducted about the LC-MS/MS profile and the biological activities of the ethanol extracts of *Z. clinopodioides* collected from Ardahan.

The aim of this study was to evaluate phenolic compounds and antioxidant potential by using DPPH free radical scavenging, ABTS cation radical scavenging, cupric reducing, and *β*-carotene bleaching activities methods, anticholinesterase, tyrosinase, and urease enzyme inhibition activities, antimicrobial activities, and anticancer potential of *Z. clinopodioides*.

MATERIALS AND METHODS

Chemicals and instruments

Chemical compositions of *Z. clinopodioides* extracts were determined by using LC–MS/MS (Shimadzu, Kyoto, Japan). A Shimadzu UV spectrophotometer and BioTek PowerWave XS microplate reader (USA) were used for the activity assays. All chemical compounds which were used in the LC-MS/MS analysis and the biological assays were purchased from Merck (Germany), Sigma (Germany); and Fluka (Germany). All solvents were of analytical grade.

Plant material

Ziziphora clinopodioides was collected from Ardahan, Turkey in July 2014 and identified by Dr. Y. Yesil. A voucher specimen was deposited in the Herbarium of Faculty of Pharmacy of Istanbul University (ISTE 116052).

Preparation of the extracts

10 g samples (aerial /root parts) were macerated in 100 mL of ethanol for 24 hours at room temperature. The extract was filtered through Whatman No 1 filter paper and the residue was re-macerated under the same condition with 100 mL of ethanol two more times. The combined filtrate was concentrated in a vacuum at 35ºC to remove the organic solvent. The extract was stored at -20ºC until ready to be used for LC–MS/MS analysis and biological activities. Dry filtrates were diluted to 250 mg/L and filtrated with 0.2 µm microfiber filter prior to LC-MS/MS analysis.

LC–MS/MS instrument and chromatographic conditions

Quantitative analyses of 37 compounds were carried out by using Shimadzu UHPLC (Nexera) coupled to a tandem MS detector. The equipment, all conditions of the method, and method validation parameters were used in the study of Yilmaz et al. (Yilmaz et al., 2018).

Total phenolic and flavonoid contents of the extracts

The colorimetric assay was carried out to determine the total phenolic content expressed as micrograms of pyrocatechol equivalents (PEs) of the extracts by using the method which has been explained in the literature in detail (Boga et al., 2016). The flavonoid contents of the extracts were measured by using the method expressed by Moreno et al., and the results were given as quercetin equivalents (QEs) (Boga et al., 2016; Moreno, Isla, Sampietro, & Vattuone, 2000).

DPPH free radical scavenging activity

The DPPH free radical scavenging potential was evaluated by using a method described by Blois (Blois, 1958; Boga et al., 2016). Absorbance at 517 nm was determined after 30 min against a blank. Inhibition % was calculated using the following equation:

DPPH scavenging effect (Inhibition, %) =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

"$A_{Control}$ is the absorbance of the control, A_{Sample} is the absorbance of the extracts or positive controls."

ABTS cation radical decolorization assay

ABTS cation radical decolorization activity of the extracts was assessed using a method informed by Re, et al (Boga et al., 2016; Re et al., 1999). The percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (methanol). Potential of the scavenging capability of $ABTS^{\bullet+}$ was calculated using the following equation:

$ABTS^{\bullet+}$ scavenging effect (Inhibition, %) =

$$\frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Cupric reducing antioxidant capacity (CUPRAC)

The cupric reducing antioxidant capacity (CUPRAC) of the extracts was determined based on the method summarised by Apak, et al. The absorbance was measured at 450 nm, and the results were given as absorbance value (Apak, Güçlü, Özyürek, & Karademir, 2004; Boga et al., 2016).

***ß*-Carotene linoleic acid test system**

The *β*-carotene linoleic acid test system was used to evaluate the antioxidant capacity of the extracts (Miller, 1971). The absorbance values of the samples and standard compounds (*α*-Tocopherol and BHT) were measured by using a 96-well microplate reader at 470 nm (BioTek Power Wave XS, USA).

"The bleaching rate (R) of *β*-carotene was calculated according to the following equation:

$$R = \frac{\ln \frac{a}{b}}{t}$$

ln is natural log, a is the absorbance at time zero, b is the absorbance at time t (120 min).

The inhibition % was calculated by using the following equation:

$$\text{Inhibition, \%} = \frac{R_{Control} - R_{Sample}}{R_{Control}} \times 100$$

Anticholinesterase activity

The acetyl- and butyryl-cholinesterase inhibitory activities were detected using a method developed by Ellman et al. (Ellman, Courtney, Andres Jr, & Featherstone, 1961).

BioTek Power Wave XS at 412 nm was used to monitor the hydrolysis of these substrates.(Boga et al., 2016). Inhibition % was calculated using the following equation:

$$\text{Inhibition, \%} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Anti-tyrosinase activity

In vitro anti-tyrosinase activity of the extracts were performed according to the method designed by Hearing and Jimenez. (Hearing & Jiménez, 1987).

Firstly, the inhibition of diphenolase function of the compounds was evaluated and *L*-DOPA was used as substrate. Tyrosinase from mushroom (E.C. 1.14.18.1) (30 U, 28 nM) was dissolved in Na-phosphate buffer (pH=6.8, 50 nM) and the compounds were added to the solution for pre-incubation at room temperature for ten minutes. To start the enzymatic reaction, 0.5 mM *L*-DOPA was added to the mixture and the change in absorbance was measured at 475 nm at 37ºC. For the positive control, kojic acid was used.

The following formula was used to calculate the percentage of all enzyme inhibitions:

$$\text{Inhibition, \%} = (A_{control} - A_{sample}) / A_{control} \times 100$$

Antiurease activity

Urease inhibition activity of the studied extracts was investigated using the protocol reported by Zahid et al. (2015). (Zahid et al., 2015).

5 µL of sample solutions (4000 ppm, in methanol) were mixed with 25 µL of urease (from *Canavalia ensiformis* type III) solution and this mixture was incubated at 30°C for 15 minutes. The substrate solution was prepared by mixing urea (100 mM) and 40 µL of phosphate buffer. The enzyme and substrate solutions then stirred and reincubated for 30 minutes. After that, 50 µL each of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 70 µL of alkali reagent (0.5% w/v sodium hydroxide NaOH and 0.1% sodium hypochlorite NaOCl) were added to the mixtures. After incubation for 50 minutes, the change in absorbance was read at 630 nm.

The following equation was used to calculate the antiurease activity:

Antiurease activity (Inhibition %) = $(A_{control} - A_{sample}) / A_{control} \times 100$

Antimicrobial activity

In vitro antibacterial activities of the *Z. clinopodioides* extracts and standard compounds were investigated by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 1997, 2006, 2010). Minimum inhibitory concentrations (MICs) of the samples were determined against *Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 4352, *Proteus mirabilis* ATCC 14153, *Candida albicans* ATCC 10231, *Candida parapsilosis* ATCC 22019, and *Candida tropicalis* ATCC 750. Minimum inhibitory concentrations (MICs) of compounds were determined by microbroth dilution technique as described by the Clinical and Laboratory Standards Institute (CLSI, 1997, 2006). Cefuroxime-sodium, cefuroxime, ceftazidime, amikacin, amphotericin B, and clotrimazole were used as standard compounds for bacteria and yeast. The antimicrobial effects of the solvents were tested against the microorganisms as a control.

Cytotoxicity assay on renal and colon cell lines

A two-day assay was used to determine the cytotoxic potential of the extracts. XTT bioassay is an in vitro antitumor colorimetric assay developed by the MTP (Molecular Targets Program), Assay Development and Screening Section, NCI (National Cancer Institute). The renal cancer cell lines (UO-31 and A498) and colon cancer cell lines (COLO205 and KM12) were used in the assay. All details of the assay was performed according to Cho et al. (Cho et al., 2017).

Metastatic potential assay

The metastatic potential of the extracts was identified by using XTT assay, developed by the MTP (Molecular Targets Program), Assay Development and Screening Section, NCI (National Cancer Institute), via comparing the effects of the extracts on high (MG63.3) and low (MG63) metastatic potential osteosarcoma cell lines. The cells were plated in tissue culture plates and allowed to attach overnight followed by 2 day treatment with the extracts. Relative cell numbers we re assessed using the XTT assay. The results were evaluated to determine which extract has activity ≤50% for MG63.3 and ≥50% for MG63, and the difference between the two values must be ≥50%.

Statistical analysis

All measurements were repeated three times. The results were evaluated using t test with Microsoft Excel and expressed as mean ± standard deviation. Differences were considered significant at $p<0.05$

RESULTS AND DISCUSSION

LC-MS/MS results

The chemical composition of the extracts is presented in Table 1. In the ethanol extracts obtained from aerial parts and roots of *Z. clinopodioides*, the major compounds were detected as quinic acid, malic acid, and rhoifolin by LC-MS/MS. The quantities of the compounds in the aerial parts extract were calculated as 9020.51±73.97 µg/g, 1972.95±22.29 µg/g, and 1972.95±22.29 µg/g, respectively. In the roots extract, the amounts of the compounds were determined as 14721.04±120.71 µg/g, 2179.04±24.62 µg/g, and 3593.31±338.13 µg/g, respectively. The current study results also showed that the extracts contain some flavonoids: hesperidin, naringenin, rutin, apigenin, and isoquercetin, as well as some phenolic acids.

Despite the fact that there are an abundance of studies focused on the chemical composition of the essential oil of *Z. clinopodioides*, to our knowledge, there is only one study about the identification of flavonoid content of the extracts obtained from *Z. clinopodioides*. According to that previous study, ten flavonoid compounds present in the extract, namely, baicalein, quercetin, hyperoside, quercetin-3-*O*-*β*-*D*-glucopyranoside, apigenin, kaempferol, chrysin, diosmin, linarin and rutin (Zhang et al., 2018). It must be noted that, the current study is an original study to contribute to the chemical profile of the species in detail. The quinic acid, malic acid, rhoifolin, hesperidin, p-coumaric acid, caffeic acid, salicylic acid, ferulic acid, chlorogenic acid, rosmarinic acid, protocatechuic acid, fumaric acid, vanillin, hesperetin, chrysin, apigetrin, nicotiflorin, naringenin, and isoquercetin were observed in *Z. clinopodioides* extracts for the first time.

Total phenolic and flavonoid content

The results of the total phenolic and flavonoid content of ethanol extracts *Z. clinopodioides* aerial and root parts are represented in Table 2. In the study of Unal et al. (2008), total phenolic compounds of the acetone, ethanol and water extracts of *Z. clinopodioides* were given as gallic acid equivalents. The highest total phenolic content was found in the acetone extract (Unal et al., 2008). The total polyphenolic and flavonoid content of petroleum ether, chloroform, ethyl acetate, n-butanol and ethanol extracts of *Z. clinopodioides* were studied in Tian et al. (Tian, Shi, Zhou, Ge, & Upur, 2011). The total polyphenolic content of the extracts were determined as gallic acid equivalents and ethyl acetate (19.27%), chloroform (4.99%), n-butanol (3.94%), ethanol (1.64%) and petroleum ether (0.23%), respectively (Tian et al., 2011). The total flavonoid content of the extracts were determined as rutin equivalents and concentrated in parts of ethyl acetate (65.61%), chloroform (14.36%) and n-butanol (10.76%) extracts (Tian et al., 2011). In the study Alp et al. (2016), the total phenolic content of essential oils of eight *Z. clinopodioides* ecotypes were investigated and determined as gallic acid equivalents. These ranged from 43.41 to 55.71 mg GAE/100 g fresh weight (Alp et al., 2016). In the study of Mahboubi et al. (2014), five plants belong to Labiatae family including *Thymus vulgaris, Thymus caramanicus, Zataria multiflora, Ziziphora clinopodioides* and *Ziziphora tenuior* hydroethanolic extracts were evaluated for antimicrobial properties and total phenolic content. The total phenolic content were determined as gallic acid equivalents and amount of total phenolics ranged from 3.785 to 10.247% of the dry extract. The total phenolic content of *Ziziphora clinopodioides* and *Ziziphora tenuior* were found to be 3.785 and 4.007%, respectively (Mahboubi, Kamalinejad, Ayatollahi, & Babaeian, 2014). Gursoy et al. studied the total phenolic content of methanol extracts of three different plants including *Z. clinopodioides* and the amount of the total phenolics was highest in *Z. clinopodioides* extract (129.55±2.26 µg/mg) in the studied plants (Gursoy et al., 2009). In the study

Table 1. Chemical compositions of *Ziziphora clinopodioides* extracts.

No	Analytes	RT[a]	Parent ion (m/z)	Daughter Ions	Ion. Mode	Quantification (µg analyte/g extract)[b]	
						ZCH	ZCR
1	Coumarin	17.40	147.05	91.0-103.2	Poz	N.D.	N.D.
2	Hesperidin	12.67	610.90	303.1-465.1	Poz	429.27±11.25	12.32±0.32
3	*p*-Coumaric acid	11.53	162.95	119.25-93.25	Neg	243.22±12.55	38.25±1.97
4	*o*-Coumaric acid	15.45	162.95	119.35-93.25	Neg	N.D.	N.D.
5	Gallic acid	3.00	168.85	125.2-79.2	Neg	N.D.	N.D.
6	Caffeic acid	8.80	178.95	135.2-134.3	Neg	272.71±9.65	27.61±0.98
7	Vanilic acid	8.57	166.90	152.25-108.25	Neg	N.D.	N.D.
8	Salicylic acid	11.16	136.95	93.3-65.3	Neg	132.42±4.36	28.56±0.94
9	Quinic acid	1.13	190.95	85.3-93.3	Neg	**9020.51±73.97**	**14721.04±120.71**
10	p-Hydroxybenzoic acid	7.39	136.95	93.3-65.3	Neg	N.D.	N.D.
11	Ferulic acid	12.62	192.95	178.3	Neg	46.16±2.28	97.79±4.83
12	Chlorogenic acid	7.13	353.15	191.2	Neg	22.03±0.15	N.D.
13	Rosmarinic acid	14.54	359	161.2-197.2	Neg	180.19±12.85	N.D.
14	Protocatechuic acid	4.93	152.95	108.3	Neg	118.15±4.86	199.48±8.20
15	Cinnamic acid	25.61	147.00	103.15-77.3	Neg	N.D.	N.D.
16	Sinapinic acid	12.66	222.95	208.3-149.2	Neg	N.D.	N.D.
17	Fumaric acid	1.48	115.00	71.4	Neg	687.11±8.52	810.45±10.05
18	Vanillin	10.87	151.00	136.3-92.2	Neg	47.42±1.32	437.01±12.24
19	Pyrocatechol	6.48	109.00	108.35-91.25	Neg	N.D.	N.D.
20	Malic acid	1.23	133.00	115.2-71.3	Neg	**1972.95±22.29**	**2179.04±24.62**
21	Syringic acid	9.02	196.95	182.2-167.3	Neg	N.D.	N.D.
22	Hesperetin	31.76	300.95	164.2-136.2	Neg	N.D.	2.09±0.12
23	Naringenin	30.68	270.95	151.2-119.3	Neg	21.47±1.12	N.D.
24	Rutin	12.61	609.05	300.1-271.1	Neg	90.85±1.44	N.D.
25	Quercetin	28.17	300.90	151.2-179.2	Neg	N.D.	N.D.
26	Quercitrin	16.41	447.15	301.15-255.15	Neg	N.D.	N.D.
27	Apigenin	31.43	268.95	117.3-151.2	Neg	108.74±7.07	N.D.
28	Chrysin	36.65	252.95	143.3-119.4	Neg	65.35±1.31	484.9±9.74
29	Liquitrigenin	25.62	254.95	119.25-135.15	Neg	N.D.	N.D.
30	Isoquercitrin	13.42	463.00	300.15-271.15	Neg	28.11±0.37	N.D.
31	Apigetrin	16.59	431.00	268.2-239.2	Neg	82.82±4.94	N.D.
32	Rhoifolin	16.11	577.05	269.2-211.15	Neg	**1044.74±98.31**	**3593.31±338.13**
33	Nicotiflorin	14.68	593.05	285.1-255.2	Neg	591.75±16.33	261.68±7.22
34	Fisetin	19.30	284.95	135.2-121.25	Neg	N.D.	N.D.
35	Luteolin	28.27	284.75	133.2-151.2	Neg	N.D.	N.D.
36	Myricetin	18.72	317.00	179.15-151.25	Neg	N.D.	N.D.
37	Kaempferol	31.88	284.75	255.1-117.3	Neg	N.D.	N.D.

[a]RT: Retention time, [b]Values in µg/g (w/w) of plant extracts. N.D: not detected; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

of Salehi et al. (2005), the total phenolic content of various extracts (methanol, water, water soluble methanol extract, water insoluble methanol extract, acetone, ethyl acetate and deodorized hot water extract) of *Ziziphora clinopodioides* subsp. *rigida* were investigated and methanol extract showed the biggest total phenolic content (174.8±1.2 gallic acid equivalents) (Salehi, Sonboli, Eftekhar, Nejad-Ebrahimi, & Yousefzadi, 2005). In this study, the total phenolic and flavonoid content

Table 2. Total phenolic and flavonoid contents of *Ziziphora clinopodioides* extracts[a].

Extracts	Phenolic content (µg PEs/mg extract)[b]	Flavonoid content (µg QEs/mg extract)[c]
ZCH	18.73±0.90	27.97±1.31
ZCR	13.67±0.73	4.74±0.22

a: Values are means ± SD of 3 parallel measurements; b: PEs: pyrocatechol equivalents (y=0,0395x + 0,0607 R^2=0,9980); c: QEs: quercetin equivalents (y=0,0325x + 0,0601 R^2=0,9984); ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

of ethanol extracts of aerial and root parts of *Z. clinopodioides* were investigated. Total phenolic content was given as pyrocatechol equivalents and total flavonoid content was given as quercetin equivalents. Results showed that the total flavonoid content (27.97±1.31 µg QE/mg extract) of the aerial parts of *Z. clinopodioides* extract was higher than the total phenolic content (18.73±0.90 µg QE/mg extract), and the total phenolic and flavonoid contents of aerial parts of *Z. clinopodioides* were higher than the root extract.

Antioxidant capacity

All literature related to DPPH free radical scavenging activity of *Ziziphora* species were given in this section. In the study of Salehi et al. (2005), the DPPH scavenging activities of essential oil and various extracts (methanol, water, water soluble methanol extract, water insoluble methanol extract, acetone, ethyl acetate and deodorized hot water extract) of *Z. clinopodioides* subsp. *rigida* were investigated and the methanol extract showed the highest free radical scavenging activity with IC_{50}:30.7±0.6 µg/mL (Salehi et al., 2005). In the study of Unal et al. (2008), chloroform, acetone, ethanol and water extracts of 25 plants including *Z. clinopodioides,* mostly used as remedies against various diseases in Turkish traditional medicine, were investigated for antimicrobial and antioxidant activities. Inhibition percentages of acetone, ethanol and water extracts of *Z. clinopodioides* were determined at 30-45% at 100 µg/mL concentration (Unal et al., 2008). Petroleum ether, chloroform, ethyl acetate, n-butanol and ethanol extracts of *Z. clinopodioides* were investigated for DPPH free radical scavenging activity in the study of Tian et al. (2011) The ethyl acetate extract showed the best activity with 34.11±0.54% at 1mg/mL concentration in all extracts and standard compounds of Vitamin C (Tian et al., 2011). *Z. capitata* methanol extract was investigated for DPPH radical scavenging activity and found IC_{50}:206.6±1.3 µg/mL value in the study of Mohammadhosseini et al. (2016) (Mohammadhosseini et al., 2016). Alp et al. studied eight ecotypes of *Z. clinopodioides* essential oils and they found DPPH free radical scavenging activity ranged from IC_{50}:3.60 to 4.20 mg/mL (Alp et al., 2016). In the other study related to the DPPH free radical scavenging activity of *Z. clinopodioides*, methanol extracts of three plants *Ziziphora clinopodioides, Cyclotrichium niveum*, and *Mentha longifolia* ssp. *typhoides* var. *typhoides* were investigated for antioxidant activity with DPPH free radical scavenging assay. *Z. clinopodioides* methanol extract had the highest activity with IC_{50}=37.73±1.18 µg/mL in the studied plants (Gursoy et al., 2009). In our study, aerial parts of *Z. clinopodioides* extract showed moderate DPPH free radical scavenging activity with IC_{50}=94.27±0.65 µg/mL value and better activity than root extract (Table 3). Our results confirmed those from the literature survey.

Table 3. DPPH free radical and ABTS cation radical activities of *Ziziphora clinopodioides* extracts.

Samples	IC_{50} values (µg/mL)*	
	DPPH Free Radical	ABTS Cation Radical
ZCH	94.27±0.65	40.69±0.19
ZCR	124.51±0.66	45.61±1.01
α-TOC	16.30±0.79	10.20±0.05
BHA	7.88±0.20	2.74±0.03
BHT	58.86±0.50	3.16±0.06

*Values expressed are means ± standard deviation of three parallel measurements; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Based on the literature review, there has not been a study about ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) assays of *Ziziphora* species previously . This is the first study of *Z. clinopodioides* ABTS cation radical scavenging activity and CUPRAC assay. Ethanol extracts of aerial and root parts of *Z. clinopodioides* have shown moderate inhibition of ABTS cation radicals with 40.69±0.19 and 45.61±1.01 µg/mL IC_{50} values, respectively (Table 3). The extracts did not show any activity in *β*-carotene linoleic acid test system. In CUPRAC assay, ethanol extracts of aerial and root parts of *Z. clinopodioides* have shown moderate inhibition with similar absorbance values that are 1.74±0.083 and 1.74±0.090 at 100 µg/mL concentration, respectively (Table 4).

Enzyme inhibition activities

Z. clinopodioides aerial parts extract showed only very low tyrosinase inhibitory activity. No activity was observed against other enzymes; including acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and urease enzymes (Table 5).

There are almost no *in vitro* anti-Alzheimer activity studies of *Ziziphora* species in the literature except the study of Ozdemir et al. (2013). In the study of Ozdemir et al., *Z. clinopodioides* extract was prepared with sodium phosphate buffer inhibited AChE of erythrocytes dose dependant manner. There was approximately 40% inhibition of AChE of erythrocytes at 200 µg/mL concentration (Ozdemir, Turkoglu, & Demir, 2013). In our study there were no acetylcholinesterase and butyrylcholinesterase inhibition activities. This difference is observed due to the variance in the chemical compositions of different extracts.

Table 4. CUPRAC activity of *Ziziphora clinopodioides* extracts*.

Samples	10 µg/mL	25 µg/mL	50 µg/mL	100 µg/mL
ZCH	0.28±0.0105	0.59±0.019	1.05±0.048	1.74±0.083
ZCR	0.32±0.074	0.53±0.0225	1.05±0.0427	1.74±0.0898
α-TOC	0.48±0.017	1.04±0.020	1.94±0.042	3.41±0.136
BHA	1.23±0.025	2.03±0.021	2.98±0.106	3.86±0.064
BHT	1.40±0.053	2.47±0.075	3.01±0.112	3.95±0.102

*The results are given as absorbance value (Values expressed are means ± standard deviation of three parallel measurements); ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 5. Enzyme inhibitory activities of *Ziziphora clinopodioides* extracts.

Samples	AChE (Inhibition %)[a]	BChE (Inhibition %)[a]	Tyr (Inhibition %)[a]	Urease (Inhibition %)[a]
ZCH	NA	NA	8.60±0.87	NA
ZCR	NA	NA	NA	NA
Galanthamine[b]	78.92±1.04	78.22±0.58	-	-
Kojic acid[b]	-	-	95.26±0.23	-
Tiyourea[b]	-	-	-	88.61±1.16

*Values expressed are means ± standard deviation of three parallel measurements; [a]200 µg/mL, [b]Standard compound , NA: Not Active, Not tested; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

According to the literature , there have been no reports about any tyrosinase and urease inhibition activities on *Z. clinopodioides* extracts. The ethanol extract of aerial parts of *Z. clinopodioides* showed very weak activity against tyrosinase with 8.60±0.87 inhibition %.

Antimicrobial activity

The MIC values of the extracts and standard compounds are summarized in Table 6. The in-vitro antimicrobial activities were evaluated against different Gram-positive and Gram-negative bacterial strains in addition to the antifungal activities. The results showed that both extracts possessed moderate activity against *C. tropicalis* with MIC values of 39.06 µg/mL. The results reveal that there is no difference in antimicrobial activity between the ethanol extracts of aerial parts and roots, as the antifungal activity of the extracts were able to induce appreciable growth inhibitory activity against *Candida* spp.

Cytotoxic activities

The cytotoxic activity results of the extracts on Renal and Colon cell lines were presented in Table 7. According to the results, the extracts did not show any activity against Renal (A498 and UO-31) and Colo (COLO205 and KM12) cell lines.

Metastatic potential

The metastatic potential result of the extracts was given in Table 8. The results showed that the extracts do not have any metastatic potential against high and low metastatic osteosarcoma cell lines (MG63.3 and MG63).

Table 6. Antimicrobial activity of *Ziziphora clinopodioides* extracts.

Microorganisms	MIC values of the extracts (µg/mL)	
	ZCH	ZCR
P. aeruginosa ATCC 27853	NA	NA
E. coli ATCC 25922	NA	NA
K. pneumoniae ATCC 4352	NA	NA
P. mirabilis ATCC 14153	NA	NA
S. aureus ATCC 29213	625	NA
S. epidermidis ATCC 12228	1250	312.5
E. faecalis ATCC 29212	1250	1250
C. albicans ATCC 10231	NA	NA
C. parapsilosis ATCC 22019	312.5	NA
C. tropicalis ATCC 750	39.06	39.06

NA: No Activity. Standards; Cefuroxime-Na: 1.2 µg/mL for *S. aureus* ATCC 29213, Cefuroxime 9.8 µg/mL for *S. epidermidis* ATCC 12228, Amikacin 128 µg/mL for *E. faecalis* ATCC 29212, Ceftazidime 2.4 µg/mL for *P. aeruginosa* ATCC 27853, Cefuroxime-Na: 4.9 µg/mL for *E. coli* ATCC 25922 and *K. pneumoniae* 4352, Cefuroxime-Na 2.4 µg/mL for *P. mirabilis* ATCC 14153, Clotrimazole 4.9 µg/mL for *C. albicans* ATCC 10231, Amphotericin B 0.5 µg/mL for *C. parapsilosis* ATCC 22019, Amphotericin B 1 µg/mL for *C. tropicalis* ATCC 750; ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 7. Cytotoxic activity results of *Ziziphora clinopodioides* extracts.

Extracts	Cell viability (%)			
	Renal Cells		Colo Cells	
	A498	UO-31	COLO 205	KM12
ZCH	84.53	80.01	77.51	>99.00
ZCR	63.32	95.94	92.79	>99.00

ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

Table 8. Metastatic potential results of *Ziziphora clinopodioides* extracts.

Extracts	Cell viability (%)	
	Osteosarcoma cells	
	MG 63.3	MG 63
ZCH	99.17	90.55
ZCR	99.33	97.33

ZCH: The ethanol extract of *Z. clinopodioides* aerial parts; ZCR: The ethanol extract of *Z. clinopodioides* root

CONCLUSION

Ziziphora clinopodioides is an edible plant which is widespread in mainly in central and eastern Turkey. It is traditionally used for its stomachic, carminative and antimicrobial activities. In this study, an LC-MS/MS analysis was performed for the first time to determine the chemical composition of the ethanol extracts of the aerial parts and the roots of the plant which was collected from Ardahan. It has been shown that the plant is rich in terms of phenolic compounds. Phenolic compounds are known to be beneficial to human health. Due to the rich mixture of these phenolic compounds, the extracts showed biological activities including anti-tyrosinase and antimicrobial activities *in vitro* as expectedly**.** *Furthermore* , the extracts have not shown any cytotoxic effects, which means it can be considered safe to use as food ingredients. In conclusion, this study provides evidence that *Z. clinopodioides* is a safe plant for consumption and has important potential medical uses that will benefit from further investigations.

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Original Article

Investigation of aloe-emodin and *Aloe vera* gel extract on apoptosis dependent pathways in leukemia and lymphoma cell lines

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ABSTRACT

Background and Aims: The present study was designed to evaluate the mechanism of cytotoxic effects of aloe-emodin relative to *Aloe vera* gel extract (AVG) on chronic myelogenous leukemia K562 and Burkitt's lymphoma P3HR-1 cell lines.
Methods: Cytotoxicity tests were assessed by tetrazolium bromide (MTT) assay. Annexin V-FITC/PI labelling was used for apoptosis/necrosis evaluation and western blotting for apoptotic molecules measurement.
Results: It was shown that, AE has cytotoxic activity againts K562 and P3HR-1 cells with an IC_{50} value of 60.9 µM and 28 µM, respectively. AVG was found cytotoxic on K562 cells with an IC_{50} value of 243.2 µg/mL and ineffective on P3HR-1 cells. The ratio of apoptotic cells (46.7%) was high in only K562 cells after AVG treatment. The percentage of apoptotic cells (K562=34.1, P3HR-1=38.8) was higher than necrotic cells (K562=11.9, P3HR-1=16.6) after AE treatment. The main apoptosis pathway in both cell lines was found to be through caspase-3 and caspase-9 activations after AE treatment, caspase-8 was also activated in K562 cells but suppressed in P3HR-1 cells. Likewise caspase-9 was activated in K562 cells after AVG treatment.
Conclusion: These results suggest that AE and AVG realized cell death activating apoptotic mechanisms in K562 and P3HR-1cells through extrinsic and intrinsic pathways.

Keywords: *Aloe vera*, aloe-emodin, cytotoxicity, leukemia, lymphoma, apoptosis, caspases

INTRODUCTION

As cancer continues to be one of the devastating illnesses of our century, the plant kingdom, with its diversity, is also researched in regard to cytotoxic and anticancer agents. It has been well-established that many of the naturally occurring phytochemicals can target multiple pathways involved in cancer cells and are considered as promising candidates for anticancer drug development. Several *in vivo* and *in vitro* studies were conducted with the *A. vera* leaf and gel extracts regarding their antitumour effects (Saito 1993; Tsuda et al., 1993; Corsi, Bertelli, Gaja, Fulgenzi & Ferrero, 1998; Akev et al., 2007). Aloe-emodin (AE) is an anthraquinone derivative purified from *A. vera* leaves, which, in addition to its well established laxative effect, has been reported to exhibit antiviral, antimicrobial, hepatoprotective and anticancer properties (Pecere et al., 2000; Mijatovic et al., 2004; Lu, Lin, Yang, Leung & Chang, 2007; He, Yan, Mo & Liang, 2008; Harlev, Nevo, Lansky, Ofir & Bishayee, 2012). Considerable attention has been given recently to the possibility of utilizing AE as a chemotherapeutic drug for the treatment of various types of cancers (Chen, Hsieh, Chang &, Chung, 2014). In a previous study undertaken in our laboratory, the anticancer effects of *Aloe vera* leaf skin and gel extracts, as well as aloe-emodin, were evaluated against B16F10 murine melanoma cells (Çandöken, Erdem Kuruca & Akev, 2017).

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The present study was undertaken in order to determine and to compare the cytotoxic effects of *A. vera* leaf gel extract (AVG) and AE on human chronic myelogenous leukemia K-562, human promyelocytic leukemia HL-60 and Burkitt's lymphoma P3HR-1 cell lines, and to understand pathways molecular of cell death mechanisms.

Usually, the stimuli which induces cell death *in vitro* is dose-dependent. At low doses, a variety of injurious stimuli such as heat, radiation, hypoxia and cytotoxic anticancer drugs can induce apoptosis, but these same stimuli can result in necrotic cell death, and be undesirable because it generates inflammation response and effect damaging neighboring cells, at higher doses. Consequently, an evaluation of cell death type is important as determination of IC_{50} values, that provide 50% inhibition of cell growth.

MATERIALS AND METHODS

Cell lines and cell culture

Chronic myelogenous leukemia K-562, acute promyelocytic leukemia HL-60 and Burkitt's lymphoma P3HR-1 cell lines were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI (Roswell Park Memorial Institute Medium; Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Capricorn FBS-12A), 100 units/mL penicillin and 100 µg/mL of streptomycin (Gibco 15140-122), in a humidified incubator containing 5% CO_2 at 37°C. The cells were sub-cultured every 2 or 3 days.

Preparation of extracts

Fresh leaves of *A. vera* (L.) Burm. f. (Xanthorreaceae) (ISTE No. 65118), cultivated in the Greenhouse of Istanbul University Alfred Heilbronn Botanical Garden, were used.

The activity of four types of extracts was investigated throughout the study. These were *A. vera* fresh leaf skin aqueous extract, *A. vera* leaf gel extract (AVG), *A. vera* fresh leaf skin methanolic extract and *A. vera* dried leaf skin methanolic extract. The preparation of the extracts was described previously (Çandöken et al., 2017).

Preparation of test materials and reference drugs

Aloe-emodin (AE; 1,8-dihydroxy-3-[hydroximethyl]-anthraquinone) was purchased from Sigma-Aldrich (St Louis, MO, cat no. A7687). *A. vera* extracts (10 mg/mL) and AE (20 mM) stock solutions were prepared in dimethyl sulfoxide (DMSO). Imatinib, which was used as a reference chemotherapeutic drug, was purchased from Santa Cruz.

MTT colorimetric assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was undertaken for cytotoxic activity (Mosmann 1983), as previously described (Çandöken et al. 2017). Varying concentrations of *A. vera* extracts, and as positive controls: AE (1.25 – 200 µM) and IM (Imatinib) (0.1 – 50 µM) were used in 10 µL/well volumes, and the assay was done in a total volume of 100 µL in microplates.

An ELISA microplate reader (Rayto RT-2100C) was used for absorbance measurement, and the percentage of viable cells (VI) was determined using the equation:

VI = (Absorbance of the treated cells ÷ Absorbance of the control cells) ×100

The cytotoxic concentrations of extracts that provide 50% inhibition of cell growth (IC_{50}) were calculated from a dose-response curve. The cytotoxic effects of *A. vera* extracts and reference drugs were evaluated by comparing the IC_{50} values of cell lines.

Flow cytometry analysis

An Annexin V-FITC/propidium iodide (PI) assay kit (Millipore) was used in order to determine normal, apoptotic, and necrotic cells as previously described (Çandöken et al. 2017).

Western blot analysis

For the apoptic protein expression analysis, cells (45 mL; 10^5 cells/mL culture medium) were seeded in 75 cm^2 cell culture treated flasks. After AE or AVG treatment, the cells were harvested and pelleted according to a previously described method. The pellets were washed with PBS, then resuspended in a 100 µl lysis buffer (1mM EDTA, 10 mM Tris-HCL, 0.5% Triton X-100, pH 8)with the addition (1:1000) of 100 nM phenylmethylsulfonyl fluoride (PMSF) followed by incubation on ice for 60 min by vortexing every 10 min. The cell lysate was centrifuged at 14 000 rpm for 10 min, and the supernatant was collected as total protein extract. The protein concentration was detected using the Bradford method. All the samples were mixed with Laemli sample buffer (2X) (Biorad), then transferred to a 80°C water bath for 15 min and stored at −80°C for later use. 10-20 µg of protein was separated on 15% SDS-PAGE gel performed at 200 mA. PVDF blotting membranes (0.45 micron) were activated in methanol for a few seconds, after electrophoresis. Blotting was performed overnight at 40 mA at 4°C using Towbin reagent, Wet/Tank Blotting Systems, BIORAD. The membranes were blocked with 5% skimmed milk powder in TBS (Sigma) containing 0.05% Tween-20 (Santa Cruze) for 1 h at RT, and then incubated with primary antibodies overnight at 4°C on a shaker at low speed. Antibodies directed against the following proteins were used in this study: actin (1:1000, Santa Cruz Biotechnology, sc-1616, goat polyclonal IgG), caspase-3 (1:200, Santa Cruz Biotechnology, sc-7148, rabbit polyclonal IgG), caspase-8 (1:200, Santa Cruz Biotechnology, sc-56070, mouse monoclonal IgG_1), caspase-9 (1:200, Santa Cruz Biotechnology, sc-7885, rabbit polyclonal IgG). The secondary antibodies used were goat anti-rabbit IgG-HRP (HRP: Horseradish Peroxidase, Santa Cruz Biotechnology, sc-2030) for caspase-3 and caspase-9, and goat anti-mouse IgG-AP (AP: Alkaline Phosphatase, Santa Cruz Biotechnology, sc-2008) for caspase-8. The fluorescent bands were visualized with the KETA Wealtec Chemiluminescence Imaging System with Magic-Chemi software, and AP detection was performed using the Novex® AP Chromogenic Substrate (Invitrogen, WP20001).

Statistical analysis

The results were statistically analyzed using the independent Student's *t*-test. Data were represented as means ± standard deviation (S.D.) and at least in triplicate. Results were considered significant with $P<0.05$ (*), $P<0.01$ (**) ve $P<0.001$ (***).

RESULTS

Cytotoxic activity

The cytotoxic effects were evaluated by comparing the IC_{50} values of all of the cell lines. Cytotoxic activity results obtained from MTT are summarized in Table 1. The data showed that AE and *A. vera* extracts had potential selective cytotoxic activity against the cells investigated. Further studies were carried out using IC_{50} concentrations as described (Mahbub et al. 2013).

The cytotoxic effects of *A. vera* extracts (62.5, 125 and 250 µg/mL) on K-562, HL-60 and P3HR-1 cells given as percentage of viable cells are shown in Figure 1. The cytotoxic efficacy was as follows: AVG >*A. vera* dried leaf skin methanolic extract > *A. vera* fresh leaf skin methanolic extract > *A. vera* fresh leaf skin aqueous extract for K-562 cells, *A. vera* fresh leaf skin methanolic extract >*A. vera* dried leaf skin methanolic extract > AVG > *A. vera* fresh leaf skin aqueous extract for HL-60 cells, whereas, P3HR-1 human lymphoma cells showed resistance to *A. vera* extracts (Table 1).

The cytotoxic effects of AE on K-562, HL-60 and P3HR-1 cells given as percentage of viable cells are shown in Figure 2. The results showed that the IC_{50} value of AE against K-562 was 60.98±0.90 µM AE. Contrarily, AE showed lower IC_{50} at 20.93±1.96 µM and 28.06±1.69 µM AE concentration against HL-60 and P3HR-1 respectively (Table 1). Anticancer drugs with limited side effects, inducing apoptosis and targeting selective cytotoxicity to the cancer cells are the drugs of choice.

The effects of IM on K-562, HL-60 and P3HR-1 cells given as percentage of viable cells are shown in Figure 3. It was shown

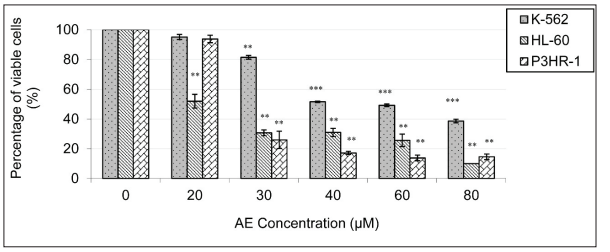

Figure 2. The effects of AE on K-562, HL-60 and P3HR-1 cells. Proliferation suppression appears to be dose dependent. Reported mean±S.D. values are from a representative trial out of two or more trials. Significant differences are indicated by *(P<0.05), **(P<0.01) ve ***(P<0.001), compared with control cells.

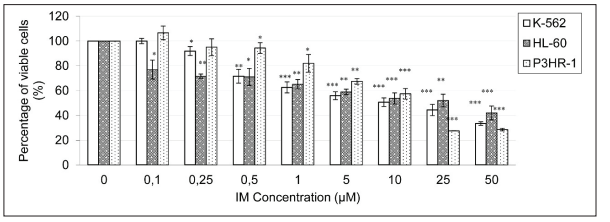

Figure 3. The effects of *Imatinib* on K-562, HL-60 and P3HR-1 cells. Proliferation suppression appears to be dose dependant. Reported mean±S.D. values are from a representative trial out of two or more trials. Significant differences are indicated by *(P<0.05), **(P<0.01) ve ***(P<0.001), compared with control cells.

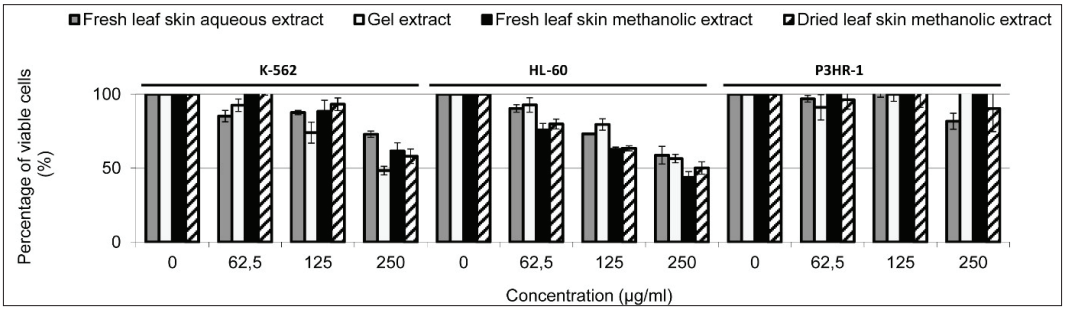

Figure 1. The effects of *A. vera* extracts on K-562, HL-60 and P3HR-1 cells. Proliferation suppression appears to be dose dependent. Reported mean±S.D. values are from a representative trial out of two or more trials.

Table 1. IC_{50} values of AE, *A. vera* extracts and IM for leukemia and lymphoma cell lines.

	IC_{50}±S.D.					
Cell line	AE (µM)	Fresh leaf skin aqueous extract (µg/mL)	Gel extract (µg/mL)	Fresh leaf skin methanolic extract (µg/mL)	Dried leaf skin methanolic extract (µg/mL)	IM (µM)
K-562	60.98±0.90	485.53±83.47	243.21±19.22	336.39±78.67	323.80±54.41	10±0.34
HL-60	20.93±1.96	290.22±47.41	289.42±18.17	204.84±2.31	229.34±20.13	25±1.28
P3HR-1	28.06±1.69	>250	>250	>250	>250	15±0.55

AE: Aloe-emodin; IM: Imatinib. K-562 human chronic myelogenous leukemia; HL-60 human acute promyelocytic leukemia; P3HR-1 human Burkitt's lymphoma.

that among leukemia cells, IM has selective cytotoxic effect on HL-60 cells at 10±0.34 µM, while it has no effect on HL-60 cells in the same concentrations with an even higher IC_{50} level such as 25±1.28 µM. The IC_{50} value of IM against P3HR-1 was 15±0.55 µM at the same time.

Annexin-V/PI apoptosis assay

To corroborate that the cytotoxic effect of K-562, HL-60 and P3HR-1 cells treated with IC_{50} values of 60 µM, 20 µM and 28 µM AE for 72 h, respectively were associated with the induction of apoptosis, an Annexin-V-FITC/PI double-staining assay was used to evaluate the percentage of apoptotic and necrotic cells. AE have apoptotic and necrotic effects on K-562 (33.99% apoptosis and 11.99% necrosis) and P3HR-1 (38.85% apoptosis and 16.66 % necrosis) cells, while treatment with AE in HL-60 cells did not induce apoptosis and necrosis in cells (Figure 4). Apoptosis and necrosis were not detected as possible mechanisms of cytotoxicity of AE in HL-60 cells.Annexin V-propidium iodide double-staining demonstrated AE potentiate apoptosis rather than necrosis in K-562, HL-60 and P3HR-1 cells.

As shown in Figure 5, AVG have apoptotic and no necrotic effects on K-562 (46.75% apoptosis and <1% necrosis) and P3HR-1 (21.49% apoptosis and <1% necrosis) cells with IC_{50} values of 250 µg/mL and 290 µg/mL AVG respectively. AVG was not assessed on the P3HR1 cells with the Annexin V-FITC/PI assay because it was ineffective.

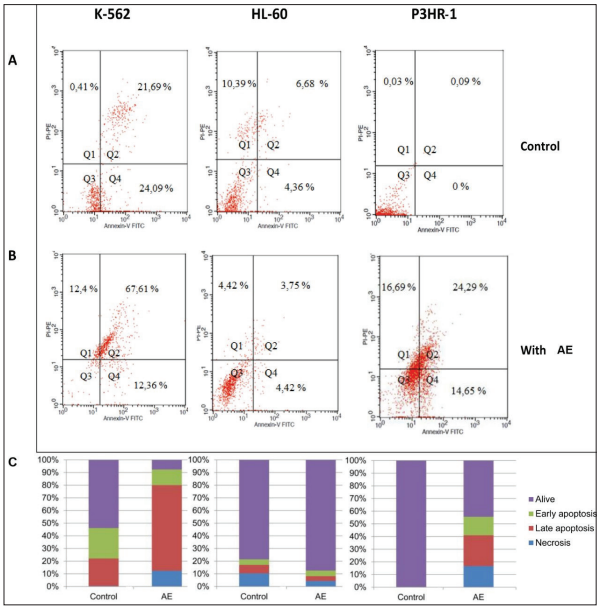

Figure 4. Flow Cytometry of K-562, HL-60 and P3HR1 cells treated with annexin V/PI. The cells after treatment with IC_{50} concentration of AE for 72 h, stained with FITC-annexin V/PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis. Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. (**A**) Control cells without the presence of aloe emodin; (**B**) Cells incubated with aloe emodin, The analysis of data from flow Cytometry was performed using the FlowJo software (**C**) Statistical analysis of alive, early apoptosis, late apoptosis and necrosis phenomena observed in aloe emodin treatment and non-treatment groups.

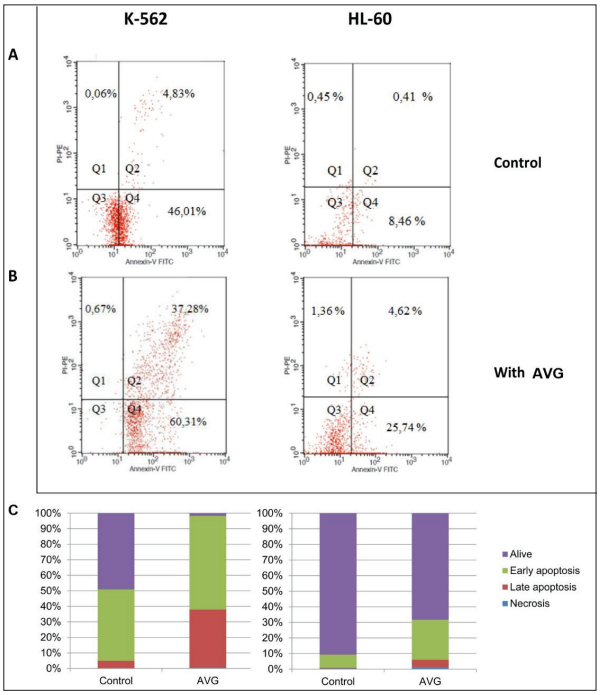

Figure 5. Flow Cytometry of K-562 and HL-60 cells treated with annexin V/PI. The cells after treatment with IC_{50} concentration of *A. vera* gel (AVG) for 72 h, stained with FITC-annexin V/PI and analyzed by FACScan flow cytometer marked for apoptosis/necrosis. Q1: Annexin V negative/PI positive; Q2: Annexin V/PI positive; Q3: Annexin V positive/PI negative; Q4: Annexin V/PI negative. (**A**) Control cells without the presence of AVG; (**B**) Cells incubated with AVG, The analysis of data from flow Cytometry was performed using the FlowJo software (**C**) Statistical analysis of early and late apoptosis phenomena observed in AVG treatment and non-treatment groups.

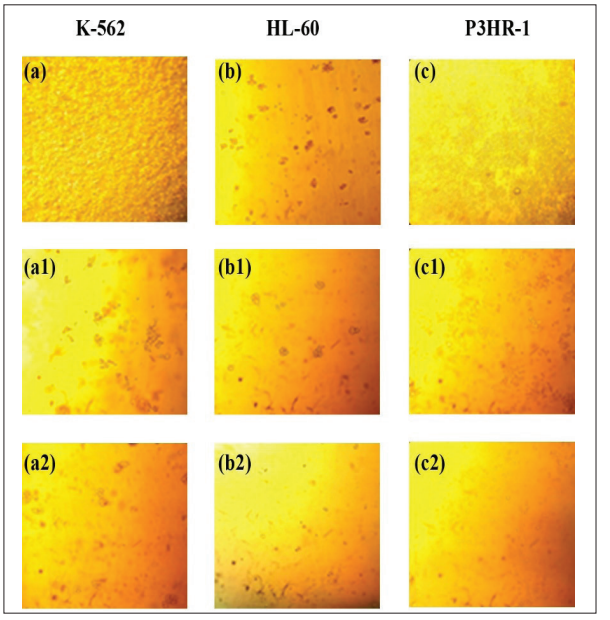

Figure 6. Inverted microscope images(X10), after treatment with the IC50 concentration for AE and AVG for 72 h. (a): K-562 cells, (b): HL-60 cells, (c): P3HR-1 cells, (a1): K-562 cells treated with AE for 72 h, (b1) HL-60 cells treated with AE for 72 h, (c1) P3HR-1 cells treated with AE for 72 h, (a2) K-562 cells treated with AVG for 72 h, (a2) HL-60 cells treated with AVG for 72 h, (b2) P3HR-1 cells treated with AVG for 72 h.

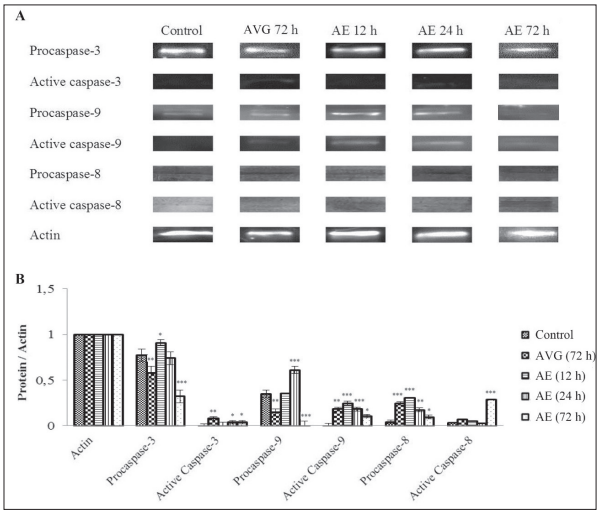

Figure 7. Western blot analysis of caspase proteins expression levels after AVG and AE treatment of K562 cells. (**A**): The K562 cells after treatment with IC_{50} concentration of AVG for 72 h and AE for 12 h, 24 h, 72 h, equal amounts of total protein were examined by western blot analysis with indicated antibodies. Actin was used as a loading control. (**B**): Band intensity was analyzed by densitometry. Fold change of protein expression levels was calculated after bands were normalized to Actin. Representative data from three independent experiments are shown. Values represent mean±SD from three independent experiments. Significant differences are indicated by *(P<0.05), **(P<0.01) ve ***(P<0.001), compared with control cells.

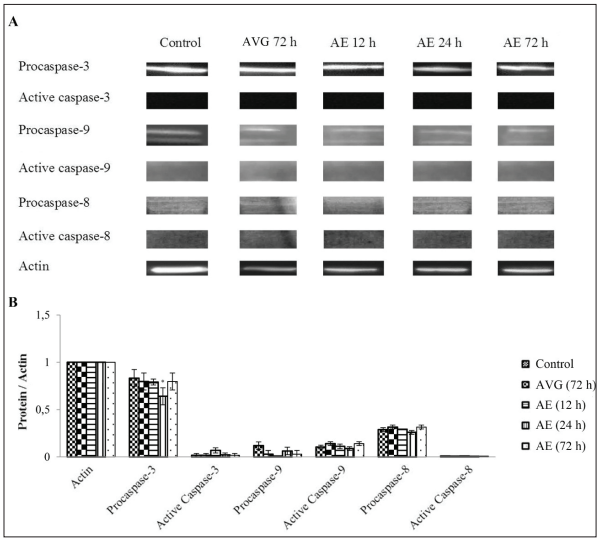

Figure 8. Western blot analysis of caspase proteins expression levels after AVG and AE treatment of HL-60 cells. (**A**): The HL-60 cells after treatment with IC_{50} concentration of AVG for 72 h and AE for 12 h, 24 h, 72 h, equal amounts of total protein were examined by western blot analysis with indicated antibodies. Actin was used as a loading control. (**B**): Band intensity was analyzed by densitometry. Fold change of protein expression levels was calculated after bands were normalized to Actin. Representative data from three independent experiments are shown. Values represent mean±SD from three independent experiments. Significant differences are indicated by *(P<0.05), **(P<0.01) ve ***(P<0.001), compared with control cells.

The morphological assessment of the effect of AE and AVG on cellular proliferation of cells was also demonstrated by Inverted microscope images (Figure 6).

Expression of apoptotic-related proteins in AVG treated K-562 cells

To understand the underlying molecular mechanisms of AE and AVG induced apoptosis in K-562 cells, several molecular markers critical for the apoptosis process were analyzed through western blotting. After exposure of K-562 cells to AE for 12, 24 and 72 hours, as shown in Figure 7, the active caspase-9 and procaspase-8 expression increased in the first 12 hours, the procaspase-8 protein was cleaved to the corresponding active forms in the 24 hours and the caspase-3 active form increased in the 72 h. The AVG treatment induces apoptotic death in K-562 cells through a caspase-dependent pathway likewise AE in the 72 hours.

As shown in Figure 8, no significant changes were observed in procaspase protein expression nor in the amount of active caspase. The AVG treatment did not induce apoptotic death in HL-60 cells through a caspase-dependent pathway likewise AE in the 72 hours.

After the P3HR-1 cells were exposed to AE for 12, 24 and 72 hours, as shown in Figure 9, procaspase-3, -9, -8 protein were

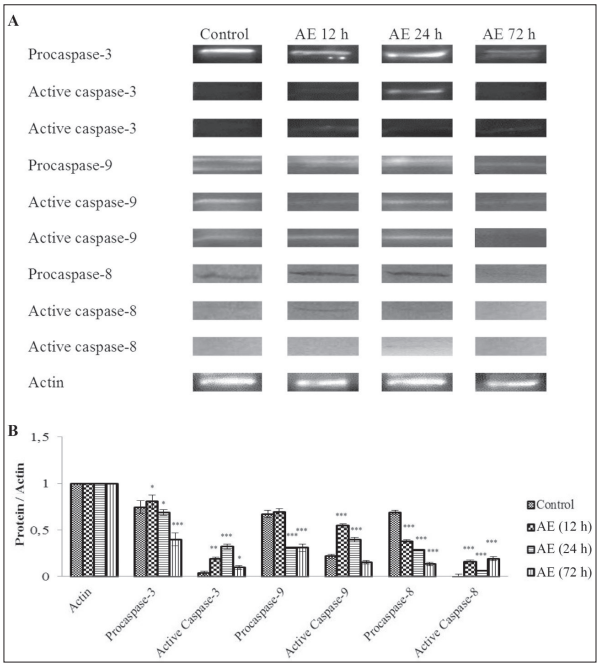

Figure 9. Western blot analysis of caspase proteins expression levels after AE treatment of P3HR-1 cells. (**A**): The P3HR-1 cells after treatment with IC_{50} concentration of AVG for 72 h and AE for 12 h, 24 h, 72 h, equal amounts of total protein were examined by western blot analysis with indicated antibodies. Actin was used as a loading control. (**B**): Band intensity was analyzed by densitometry. Fold change of protein expression levels was calculated after bands were normalized to Actin. Representative data from three independent experiments are shown. Values represent mean±SD from three independent experiments. Significant differences are indicated by *(P<0.05), **(P<0.01) ve ***(P<0.001), compared with control cells.

cleaved to the corresponding active forms in the first 12 hours. These results indicate that AE treatment induces apoptotic death in P3HR-1 cells through a caspase-dependent pathway.

DISCUSSION

Among the various known therapeutic effects of *Aloe vera,* studies have shown that preparations of the plant leaves have the ability to prevent the growth or to regress certain tumours (Akev et al., 2015). Considerable attention has been given recently to the possibility of utilizing AE, an anthraquinone purified from *A. vera* leaves, as a chemotherapeutic drug (Chen et al., 2014). Some researchers claim that the synergistic effect of the compounds contained in plant extracts should be preffered to purified compounds (Williamson, 2001).

A number of studies have demonstrated that AE is capable of inducing apoptotic cell death in various cancer cells such as neuroectodermal cancer cells (Pecere et al., 2000), hepatoma (Kuo, Lin & Lin, 2002), gastric cancers (Chen, Lin, Chang, Fang & Lin, 2007; Qin et al., 2010), bladder carcinoma (Lin et al., 2006), glioma cells (Acevedo-Duncan, Russel, Patel & Patel, 2004) or squamous cell cancer (Chiu et al., 2009). Some of the anticancer mechanisms were also related to cell cycle arrest (Chen et al., 2004; Guo et al., 2007; Chiu et al., 2009) or DNA damage through oxidative stress (Lee, Lin, Yang, Leung & Chang, 2006).

There are also a few studies undertaken on leukemia cells (Tabolacci et al., 2011). The IC_{50} value of 29 µM was found for aloe-emodin in human K562 leukemia cell line (Harlev et al., 2012). Mahbub et al. (2013) found an IC_{50} value of 500 µM for emodin in K562 cells. In our study, this value was found to be 60.98±0.90 µM. Chen et al. (2004) reported an IC_{50} value of AE for HL-60 / ADR of 5.79 µmol / L. In our study, the IC_{50} value of AE for HL-60 was found to be 20.93±1.96 µM. No literature was found for AE treatement with P3HR-1 human lymphoma cells. In our study, the IC_{50} value of AE for P3HR-1 human lymphoma cells was 28.06±1.69 µM.

Imatinib mesylate (IM), a specific Bcr-Abl tyrosine kinase inhibitor, has been highly successful in the treatment of chronic myelogenous leukemia (Gu, Santiago & Mitchell, 2005). Therefore, it was chosen as a reference drug in the present study. The IC_{50} value of IM was reported to be 10.49±1.24 µM in K562 human chronic myeloid leukemia cells (Wu, Huang & Ma, 2013). Similarily, in our study, this value was found to be 10±0.34 µM. for the same cell line. The IC_{50} value of IM for HL-60 human promyelocytic leukemia cells was > 8 µM for 48 h (Gu et al. 2005). In our study, the IC_{50} value of IM for HL-60 human promyelocytic leukemia cells was found to be 25±0.51 µM. There has been no published literature on the IC_{50} value of IM for P3HR-1 human lymphoma cells. In our study, the IC_{50} value of IM for P3HR-1 cells was found to be 15±5.5 µM.

Induction of apoptosis is an important mechanism of chemoprevention and chemotherapy for cancer. The variety of injurious stimuli such as heat, radiation, hypoxia and anticancer drugs can induce apoptosis, but these same stimuli can result in necrotic cell death, undesirable because it generates inflammation response. The apoptotic and necrotic cell death are directly related to the drug concentration because drug treatment in high concentration causes necrosis, and also low concentration stimulates apoptosis. To determine whether the inhibition of cell proliferation by extracts from *A. vera* was due to the induction of apoptosis, we assessed the latter with the Annexin V-FITC/PI method. Therefore, we preferred the percentage of apoptosis as a criterion in evaluation of cell death type. IC_{50} values (concentration that provide 50% inhibition of cell growth) were used to assess the cytotoxic effects of extracts and comparing to reference drugs. Among all the extracts tested, *A. vera* gel extract was found to be the most effective on the tested cells, with the lowest IC_{50} values. Therefore, apoptosis and necrosis were measured in cells treated with AVG or AE in the doses according to IC_{50} values. Flow Cytometry images clearly showed apoptosis and necrosis changes in the treated cells.

Chen et al., (2004) reported that aloe-emodin inhibited cell proliferation in human promyelocytic leukemia HL-60 cells, caused cell cycle involvement in G2 / M phase and showed apoptotic activity. They found that morphological changes in the cells were observed in 10 - 25 µM aloe-emodin applications. In the determination of the apoptotic pathway, the effect of 10 µM aloe-emodin dose on the expression of caspase-3, caspase-8 and caspase-9 expressions in cells at different times (12, 24, 48, and 72 h) was determined (Chen et al., 2004).

In a study conducted by Mahbub et al. (2013), the apoptotic effect of AE on HL-60 and K562 cells was performed by Hoechst 33342 with DNA staining and caspase-3 activity by Flow Cytometry. HL-60 cells were found to be more sensitive than K562 cells to AE treatment and also the dose of 50 µM AE induced significant induction of caspase 3 activity in both cell lines. Contrary to this study, in our study, Western Blot studies conducted with AE at IC_{50} doses on HL-60 cells, did not show similar AE-induced caspase-3 activity. This result was found to be compatible with our Annexin V / PI test of AE for HL-60 cells test results (0.54% apoptosis). The apoptotic effect of *A. vera* gel (21.49% apoptosis) observed in the Annexin V / PI test for HL-60 cells could not be shown in Western Blot experiments. *A. vera* gel treatment did not induce apoptotic death in HL-60 cells through a caspase-dependent pathway likewise AE in the 72 hours. Here, the mechanism is caspase-dependent apoptosis, whereas caspase-independent apoptosis is also known. Accordingly, the apoptosis observed in HL60 cells of *A. vera* gel may be caspase-independent. Whereas IC_{50} doses of 60 µM AE treatment induce apoptotic death in K-562 cells through a caspase-dependent pathway likewise AVG in the 72 hours.

Cytotoxicity and Western Blot studies with *A. vera* extracts or aloe-emodin for P3HR-1 Burkitt's lymphoma cells were not found in the literature. In our study, the Annexin V / PI study with AE for P3HR-1 cells concluded that the apoptotic protein expression (increased active caspase-3 expression) was compatible with the end result (38.85% apoptosis for aloe- emodin). Our results suggested that caspase-3 was at the top of the hierarchy of the caspase cascade and is an initiator caspase to be evoked. This activation occured as early as 12 h af-

ter AE treatment in P3HR-1 cells, whereas caspase-9 showed activation after 24 h. There was no significant change in the expression of active caspase-8 until the end of 72 h, whereas an increase in active caspase-9 was observed in the 24 h experiment.

As a conclusion we can say that their selective cytotoxic and apoptotic effects make *A. vera* gel and aloe-emodin promising drugs for alternative cancer therapy.

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Original Article

Synthesis and antimicrobial activity evaluation of new hydrazide-hydrazones derived from 1,2,4-triazole

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ABSTRACT

Background and Aims: The aim in this study was to synthesize new 2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4*H*-1,2,4-triazol-3-yl]sulfanyl]-*N'*-[(substituted phenyl/furanyl)methylidene]acetohydrazides and screen for their *in vitro* antimicrobial activity.
Methods: Novel compounds (**6a-g**) were synthesized starting with furan-2-carbohydrazide (**1**) using five step reactions. The structures of the the resulting compounds were characterized by IR, ^{1}H-NMR and elemental analysis. All compounds were evaluated for antibacterial and antifungal activities against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258, *Candida parapsilosis* ATCC 22019, *Microsporum gypseum* NCPF 580, *Trichophyton mentagrophytes* var. *erinacei* NCPF 375 and *Trichophyton tonsurans* NCPF 245 using a microbroth dilution method.
Results: The biological assay results showed that all of the compounds displayed varying degrees of antimicrobial activity in this series.
Conclusion: The preliminary results revealed that some of the new synthesized derivatives exhibited promising antimicrobial activities. Further investigation may be completed on similar molecules in the future.

Keywords: Synthesis, hydrazide-hydrazone, antibacterial activity, antifungal activity

INTRODUCTION

1,2,4-Triazole derivatives have a high potency for different biological activities, such as anticancer (Boraei et al., 2019; El-Sherief et al., 2018), antiproliferative and anti-tubulin (Mustafa et al., 2019; Mustafa et al., 2017), antibacterial (Özkırımlı et al., 2009; Turan-Zitouni et al., 2005; Sztanke et al., 2008; Demirbaş et al., 2004; Ulusoy et al., 2001*a*), analgesic (Turan-Zitouni, Kaplancıklı, Erol, & Kılıç, 1999), anti-inflammatory (Tozkoparan, Gökhan, Aktay, Yeşilada, & Ertan, 2000) and antitumour (Demirbaş et al., 2004; Al-Soud et al., 2004; Holla et al., 2003) properties. Moreover, hydrazide-hydrazone derivatives have been reported to posses antimicrobial (Rollas, Güleman, & Erdeniz, 2002), antituberculosis (Koçyiğit-Kaymakçıoğlu et al., 2006; Imramovsky et al., 2007), herbicidal (Weng, Huang, Zeng, Deng, & Hu, 2012), antiviral (Završnik et al., 2011), analgesic-anti-inflammatory (Salgın-Gökşen et al., 2007), anticonvulsant (Dimmock, Vashishtha, & Stables, 2000) and anticancer (Savini et al., 2004) activities.

In continuation with our previous work in the antimicrobial activity research area, in the present study we have demonstrated the structural diversity of 1,2,4-triazoles as potent antimicrobial agents (Ergenç et al., 1996; İlhan et al., 1996; Günay et al., 1999;

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Ulusoy et al., 2001*a*; 2001*b*; 2003; Ulusoy Güzeldemirci et al., 2010; 2013), we report here the synthesis and investigation of antimicrobial activity of new 1,2,4-triazole compounds.

MATERIALS AND METHODS

Chemistry

All reagents for synthesis were commercially available. The melting points were determined on a Büchi 530 melting point apparatus (Flawil, Switzerland) in open capillary method and are uncorrected. Elemental analyses were analysed on a Carlo Erba 1106 elemental analyzer (Milano, Italy). IR spectra were obtained on KBr discs, using a Perkin Elmer 1600 FT-IR spectrophotometer (Waltham, MA, USA). ^{1}H NMR (DMSO-d_6/TMS) spectra were measured on VarianUNITY INOVA (500 MHz) spectrophotometer.

General procedure for the preparation of 2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]acetohydrazide (5)

Compound **5** was *prepared from* furan-2-carbohydrazide (**1**) using four step reactions *according to literature* procedures (Çapan et al., 1990-92; Çapan et. al., 1993; Ulusoy et al., 2001*b*).

General procedure for the preparation of 2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(substituted phenyl/furanyl)methylidene]acetohydrazides (6a-g)

A solution of **5** (0.005 mol) and the appropriate aromatic aldehyde (0.005 mol) in ethanol (30 mL) was heated under reflux for 3h. The precipitate thus obtained was filtered and purified either by washing with hot ethanol or recrystallization from ethanol.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(3-methoxyphenyl)methylidene]acetohydrazide (6a)

Yield 70%; mp 108-109°C; IR(KBr) υ cm^{-1}: 3440 (NH), 1676 (C=O), 1576, 1494, 1431 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 11.72; 11.64 (2s, 1H, CONH), 8.15; 7.96 (2s, 1H, N=CH), 7.75 (d, *J*=1.95 Hz, 1H, furan C_5-H), 7.69-7.65 (m, 2H, ClPh$C_{2,6}$-H), 7.58-7.54 (m, 2H, ClPh$C_{3,5}$-H), 7.35-7.20 (m, 3H, arylidene $C_{2,5,6}$-H), 7.00-6.96 (m, 1H, arylidene C_4-H), 6.52 (dd, *J*=3.42; 1.95 Hz, 1H, furan C_4-H), 6.29 (d, *J*=3.42 Hz, 1H, furan C_3-H), 4.45; 4.05 (2s, 2H, SCH_2), 3.77 (s, 3H, OCH_3). Anal. Calcd. for $C_{22}H_{18}ClN_5O_3S.1.5\ H_2O$: C, 53.38; H, 4.27; N, 14.15; Found: C, 53.47; H, 4.07; N, 14.27%.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(4-chlorophenyl)methylidene]acetohydrazide (6b)

Yield 59%; mp 119-120°C; IR(KBr) υ cm^{-1}: 3446 (NH), 1675 (C=O), 1610, 1559, 1514 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 11.77; 11.68 (2s, 1H, CONH), 8.69; 8.18 (2s, 1H, N=CH), 7.88 (d, *J*=1.96 Hz, 1H, furan C_5-H), 7.75-7.46 (m, 8H, ClPh), 6.53 (dd, *J*=3.42; 1.95 Hz, 1H, furan C_4-H), 6.29 (d, *J*=3.42 Hz, 1H, furan C_3-H), 4.44; 4.05 (2s, 2H, SCH_2). Anal. Calcd. for $C_{21}H_{15}Cl_2N_5O_2S.H_2O$: C, 51.43; H, 3.49; N, 14.28; Found: C, 51.22; H, 3.27; N, 13.66%.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(4-bromophenyl)methylidene]acetohydrazide (6c)

Yield 79%; mp 124-125°C; IR(KBr) υ cm^{-1}: 3440 (NH), 1676 (C=O), 1621, 1582, 1479 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 11.78 (s, 1H, CONH), 8.68; 8.16 (2s, 1H, N=CH), 7.80 (s, 1H, furan C_5-H), 7.75-7.53 (m, 8H, ClPh, BrPh), 6.52 (dd, *J*=3.41; 1.47 Hz, 1H, furan C_4-H), 6.29 (d, *J*=3.41 Hz, 1H, furan C_3-H), 4.44; 4.05 (2s, 2H, SCH_2). Anal. Calcd. for $C_{21}H_{15}BrClN_5O_2S.H_2O$: C, 47.15; H, 3.20; N, 13.09; Found: C, 47.22; H, 2.64; N, 12.72%.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(4-carboxyphenyl)methylidene]acetohydrazide (6d)

Yield 74%; mp 265-266°C; IR(KBr) υ cm^{-1}: 3487, 3189 (OH/NH), 1671 (C=O), 1603, 1567, 1494 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 11.86; 11.77 (2s, 1H, CONH), 8.23; 8.05 (2s, 1H, N=CH), 7.99-7.96 (m, 2H, arylidene $C_{3,5}$-H), 7.81-7.74 (m, 3H, arylidene $C_{2,6}$-H, furan C_5-H), 7.69-7.66 (m, 2H, ClPh$C_{2,6}$-H), 7.58-7.54 (m, 2H, ClPh$C_{3,5}$-H), 6.52 (dd, *J*=3.42; 1.95 Hz, 1H, furan C_4-H), 6.29 (d, *J*=3.42 Hz, 1H, furan C_3-H), 4.47; 4.07 (2s, 2H, SCH_2). Anal. Calcd. for $C_{22}H_{16}ClN_5O_4S.1.5\ H_2O$: C, 51.91; H, 3.76; N, 13.76; Found: C, 51.52; H, 3.59; N, 13.88%.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl]sulfanyl]-N'-[(2,6-dichlorophenyl)methylidene]acetohydrazide (6e)

Yield 75%; mp 228-229°C; IR(KBr) υ cm^{-1}: 3400 (NH), 1684 (C=O), 1610, 1579, 1494 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 12.01; 11.87 (2s, 1H, CONH), 8.38; 8.24 (2s, 1H, N=CH), 7.74 (d, *J*=0.98 Hz, 1H, furan C_5-H), 7.71-7.65 (m, 2H, ClPh$C_{2,6}$-H), 7.57-7.53 (m, 3H, ClPh$C_{3,5}$-H, arylidene C_4-H), 7.46-7.39 (m, 2H, arylidene $C_{3,5}$-H), 6.52 (dd, *J*=3.42; 1.95 Hz, 1H, furan C_4-H), 6.28 (d, *J*=3.42 Hz, 1H, furan C_3-H), 4.38; 4.08 (2s, 2H, SCH_2). Anal. Calcd. for $C_{21}H_{14}Cl_3N_5O_2S.H_2O$: C, 48.05; H, 3.07; N, 13.34; Found: C, 48.69; H, 2.71; N, 13.66%.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(3,4-dichlorophenyl)methylidene]acetohydrazide (6f)

Yield 63%; mp 158-159°C; IR(KBr) υ cm^{-1}: 3400 (NH), 1676 (C=O), 1494, 1473 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 11.88; 11.77 (2s, 1H, CONH), 8.67; 8.15 (2s, 1H, N=CH), 7.95-7.54 (m, 8H, ClPh, arylidene $C_{2,5,6}$-H, furan C_5-H), 6.51 (dd, *J*=3.41; 1.47 Hz, 1H, furan C_4-H), 6.27 (d, *J*=3.42 Hz, 1H, furan C_3-H), 4.44; 4.05 (2s, 2H, SCH_2). Anal. Calcd. for $C_{21}H_{14}Cl_3N_5O_2S.H_2O$: C, 48.05; H, 3.07; N, 13.34; Found: C, 47.98; H, 2.82; N, 12.74.

2-[[4-(4-chlorophenyl)-5-(furan-2-yl)-4H-1,2,4-triazol-3-yl] sulfanyl]-N'-[(5-nitrofuran-2-yl)methylidene]acetohydrazide (6g)

Yield 51%; mp 190-191°C; IR(KBr) υ cm^{-1}: 3096 (NH), 1683 (C=O), 1610, 1563, 1472 (C=N/C=C); ^{1}HNMR (DMSO-*d6*) δ (ppm): 12.11; 11.99 (2s, 1H, CONH), 8.70; 8.16 (2s, 1H, N=CH), 7.81-7.44 (m, 6H, ClPh, furfurylidene C_4-H, furan C_5-H), 7.22, 7.20 (2d, 1H, *J*=3.90; 3.91 Hz furfurylidene C_3-H), 6.52; 6.48 (2dd, 1H, *J*=3.41; 1.95, 3.42; 1.47 Hz furan C_4-H), 6.30; 6.22 (2d, *J*=2.93; 3.41 Hz, 1H, furan C_3-H), 4.31; 4.08 (2s, 2H, SCH_2). Anal. Calcd. for $C_{19}H_{13}ClN_6O_5S.1/2H_2O$: C, 47.35; H, 2.92; N, 17.44; Found: C, 46.91; H, 2.47; N, 17.91%.

Microbiology

The tested compounds were dissolved in DMSO at a stock concentration of 3200 µg.cm^{-3}. The final required concentrations were prepared with RPMI 1640 medium for *Candida* species

and dermatophytes and with Mueller-Hinton broth of bacteria. The last DMSO concentration was reduced to 1%.

Antibacterial activity

This was performed according to the literature method (Wayne, 2005).

Antifungal activity

Antifungal activity for Candida species

This was applied according to the literature (Wayne, 2002*a*).

Antifungal activity for dermatophytes

The microdilution method was used according to the literature (Wayne, 2002*b*; Fernández-Torres, 2002).

RESULTS AND DISCUSSION

4-(4-Chlorophenyl)-2,4-dihydro-5-(2-furyl)-3*H*-1,2,4-triazole-3-thione (**3**) was prepared from furan-2-carbohydrazide (**1**) with two-step synthesis (Çapan, Ergenç, & Ötük, 1990-92). Treatment of compound (**3**) with ethyl bromoacetate gave an ester derivative (**4**), which yielded a hydrazide compound (**5)** on reaction with hydrazinium hydroxide. Condensation of **5** with substituted aromatic aldehydes gave **6a**–**g**. *The synthesis of* the title compounds is shown in Figure 1. IR and ^{1}H NMR data confirmed the proposed structures. IR spectra showed the NH bands of **6a**–**g** in the 3487–3096 cm^{-1} regions. The CO groups of **6a**-**g** absorbed in the 1684–1671 cm^{-1} regions, respectively. The other bands which appeared in the spectra of **6a**–**g** in the 1621–1431 cm^{-1} region were attributed to the exocyclic C=N group. The ^{1}H NMR spectra of **6a**–**g** revealed the presence of two isomers in DMSO-*d6* as supported by the NH, N=CH, and SCH_2 protons resonating as double singlets at about 12.11-11.64, 8.70–7.96 and 4.47–4.05 ppm. It is supposed that the N=CH double bond limits rotation and causes the formation of *E* and *Z* isomers with the *E* isomer dominating (Çapan, Ulusoy, Ergenç, & Kiraz, 1999).

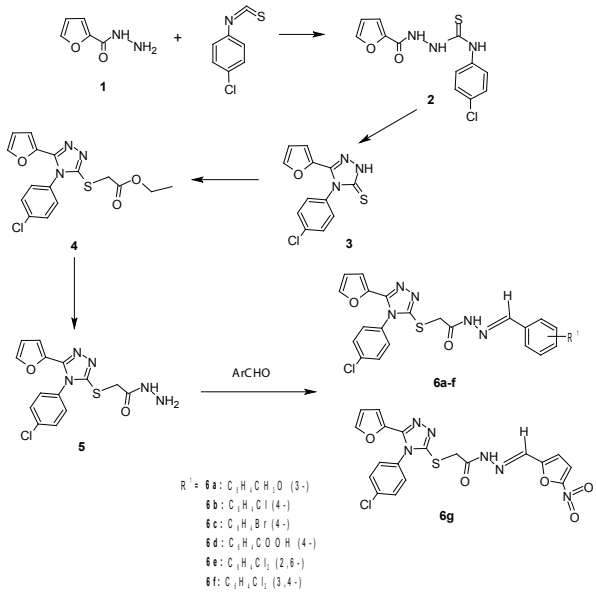

Figure 1. Synthetic route of compounds **6a-g**.

The newly synthesized compounds **6a**-**g** were evaluated for *in vitro* antibacterial activity against *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853, and for antifungal activity against *Candida albicans* ATCC 10231, *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019, *Microsporum gypseum* NCPF 580 *T. var. erinacei* NCPF 375 and, *T. tonsurans* NCPF 245 using the microbroth dilution method (Wayne, 2005) (Tables 1 and 2).

Table 1. Antibacterial activity of compounds 6a-g (MIC ug/mL)

Comp.	Microorganisms*		
	A	B	C
6a	64	64	>64
6b	>64	>64	>64
6c	64	64	>64
6d	64	64	64
6e	64	>64	>64
6f	>64	>64	>64
6g	32	64	>64
Levofloxacin	0.12	0.5	0.015

***A=** *S. aureus* ATCC 29213, **B=** *E. coli* ATCC 25922, **C=** *P. aeruginosa* ATCC 27853

Table 2. Antifungal activity of compounds 6a-g (MIC ug/mL)

Comp.	Microorganisms*					
	A	B	C	D	E	F
6a	32	32	32	32	16	16
6b	64	64	>64	16	16	8
6c	64	64	64	16	16	16
6d	32	64	32	16	16	8
6e	>64	>64	>64	16	16	8
6f	64	64	64	64	32	32
6g	64	32	32	16	16	16
Itraconazole	0.12	0.06	0.12	n.t.	n.t.	n.t.
Amphotericin B	n.t.	n.t.	n.t.	0.5	0.5	0.25

***A=** *C. albicans* ATCC 10231, **B=** *C. krusei* ATCC 6258, **C=** *C. parapsilosis* ATCC 22019, **D=** *M. gypseum* NCPF 580, **E=** *T. mentagrophytes var. erinacei* NCPF 375, **F=** *T. tonsurans* NCPF 245

5-Nitrofuryl substituted compound **6g**, showed the highest antibacterial activity against *S. aureus* ATCC 29213 (MIC=32 μg/cm^{-3}) (Table 1). Additionally, derivatives **6b** (R^1=4-ClPh), **6d** (R^1=4-COOHPh) and **6e** (R^1=2,6-$(Cl)_2$Ph), were most active against *T. tonsurans* NCPF 245 (MIC=8 μg/cm^{-3}) (Table 2). Compounds **6b**, **6c** (R^1= 4-BrPh), **6d**, **6e** and **6g** showed the highest effect against *M. gypseum* NCPF 580 (MIC=16 μg/cm^{-3}). Compounds **6a** (R^1=3-CH_3OPh), **6b**, **6c**, **6d**, **6e** and **6g** also showed the highest activity against *T. mentagrophytes var. erinacei* NCPF 375 (*MIC*=16 μg/cm^{-3}).

CONCLUSION

A new series of hydrazide-hydrazone compounds derived from 1,2,4-triazole (**6a**-**g**) were obtained from furan-2-carbohydrazide. The activity results indicated that some of the title compounds showed promising antimicrobial activity.

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Original Article

Studies on the formulation optimization and controlled ionic gelation of chitosan nanoparticles using TPP-HP-β-CD inclusion complex

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ABSTRACT

Background and Aims: Ionic gelation strategy is the most common method used for the preparation of chitosan nanoparticles to obtain controlled drug delivery. Although it is a convenient and easy method, it is highly related with particle aggregation, high polidispersity index and insufficient physical/chemical stability. The aim of this study was the development of chitosan nanoparticles using tripolyphosphate-hydroxy propyl β-cyclodextrin or tripolyphosphate-sulfobutyl ether β-cyclodextrin inclusion complex as an alternative to TPP, and hence to increase physical stability, reduce polidispersity index and develop a stable nanocarrier for drug delivery purposes.

Methods: The nanoparticles were prepared with the ionic gelation technique. The effects of chitosan percent, pH, and chitosan/tripolyphosphate ratio were investigated to find out the optimum nanoparticles in terms of particle size, polidispersity index and zeta potential. After determining the conditions for the tripolyphosphate-chitosan nanoparticles, the nanoparticles were prepared using tripolyphosphate-hydroxypropyl β-cyclodextrin or tripolyphosphate-sulfobutyl ether β-cyclodextrin to make a comparison with the nanoparticles which were prepared using tripolyphosphate.

Results: The chitosan/tripolyphosphate-hydroxypropyl β-cyclodextrin nanoparticles were successfully formulated with 178 ± 84.1 nm particle size, 0.310±0.0134 PDI, 31.2±4.68 mV zeta potential. The interday changes in the measured characteristics were minimized for chitosan/tripolyphosphate-hydroxypropyl β-cyclodextrin nanoparticles as intended.

Conclusion: CS/TPP-HP-β-CD nanoparticle formulation with particle size below 200 nm, high zeta potential and increased physical stability nanoparticles would offer a promising approach especially for hydrophobic drugs to improve their stability, solubility, encapsulation efficiency and in vivo bioavailability.

Keywords: Chitosan nanoparticles, controlled ionic gelation, cyclodextrins

INTRODUCTION

In past decades, many efforts have been made to obtain a controlled and targeted release of drugs with micro and nano sized drug delivery systems. Due to their small size and large surface area, drug nanoparticles are superior to conventional drugs, with the ability to increase solubility, and hence improve bioavailability, providing a controlled release and reduced side effects. No doubt, advances in polymer science also promoted the advancement of drug-delivery technology. Among all nanoparticulate drug delivery approaches, polymeric nanoparticles have attracted significant attention since they are biodegradable, biocompatible, relatively easy to prepare and suitable for a variety of chemical drug classes and dosage forms (Crucho & Barros, 2017; Kumari, Yadav & Yadav, 2010; Rizvi & Saleh, 2018).

Polymeric nanoparticles are mainly prepared with biodegradable polymers, either synthetic or natural ones. Poly (lactide) (PLA), poly (lactide-co-glycolide) copolymers (PLGA), poly (ε-caprolactone) (PCL) and poly(amino acids) are the most common synthetic

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polymers, while chitosan (CS), alginate, gelatin, and albumin CS-based are examples of natural polymers (Elsabahy & Wooley, 2012; Mohammed, Syeda, Wasan & Wasan, 2017). CS is a very well established chitin derivative biodegradable polymer, which is composed of β-1, 4 linked 2 amino-2-deoxy-glucopyranose and 2-acetamido-2-deoxy-B-d-glucopyranose residues. It is cationic, highly basic, mucoadhesive biocompatible and approved by the FDA for tissue engineering and drug delivery purposes (Ahmed & Aljaeid, 2016; Mohammed et al., 2017). Since the CS nanoparticles were first introduced in 1994 by Ohya and colleagues, as a carrier for 5-FU, there has been tremendous research on CS nanoparticles for various drugs/proteins, application routes and preparation techniques (Grenha, 2012, Ohya, Shiratani, Kobayashi & Ouchi, 1994) Emulsion cross-linking, coacervation/precipitation, spray-drying, emulsion-droplet coalescence, the ionic gelation method, and the reverse micellar method are the methods used for CS nanoparticle prepration (Agnihotri, Mallikarjuna & Aminabhavi, 2004).

The ionic gelation strategy is one of the methods that has been extensively used for the preparation of CS nanoparticles to obtain controlled drug delivery. Nanoparticle formation occurs with the interactions between the positively charged CS chains and polyanions of cross-linkers employed such as tripolyphosphate (TPP). Despite its convenience and abstinence of organic solvents, it is generally known that the method is conducted with particle aggregation, a high polidispersity index and insufficient pysical/chemical stability. The main reason for this problem could be undesired intra and intermolecular cross-linking between TPP and CS (Agarwal, Shrivastav, Pandey, Das & Gaur, 2018; Calvo et al., 1997, Pant & Negi, 2018).

Cyclodextrins (CDs) are cyclic oligosaccharides with a hyrophobic central cavity and hydrophilic outer surface. They are widely utilized for improving solubility by forming inclusion or non-inclusion complexes and enhancing oral absorption/bioavailability of hyrophobic drugs (Agardan et al., 2016; Loftsson & Duchene, 2007; Mutlu Ağardan et al., 2014).

In this study, it was considered to use CD complex with TPP and thus controlling ionic gelation to obstruct undesired ionic interactions between cationic CS and anionic TPP. For this reason, two CD derivatives: 2-Hydroxypropyl-β-CD (HP-β-CD) and sulfobutyl ether β-CD (SBE-β-CD) were chosen to prepare complexes with TPP. HP-β-CD is a neutral derivative, SBE-β-CD is an anionic CD derivative. In addition, the effects of pH and CS/TPP ratio were also investigated to optimise nanoparticle formation. The stability of nanoparticles following preparation was compared with CS-TPP nanoparticles, and optimum formulation parameters were determined for further drug delivery studies.

MATERIALS AND METHODS

Low molecular weight CS (LWCS) (20–30 cps and ≥75% deacetylation) and TPP were purchased from Sigma Aldrich Ltd (USA). HP-β-CD and SBE-β-CD were purchased from Cylolab, Hungary.

Preparation of TPP- HP-β-CD and TPP-SBE-β-CD inclusion complexes

TPP-HP-β-CD or TPP-SBE-β-CD complexes were prepared with a solution-ultrasonic method (Pant & Negi, 2018). TPP and HP-β-CD or SBE-β-CD were dissolved in distilled water in 1:1 molar ratio and the solution was kept in an ultrasonic bath at room temperature for 1h. Then, the solutions were filtered through 0.45 µm cellulose acetate membrane filter and the filtrates were frozen at -80°C for 30 min prior to lyophilization. The frozen samples were lyophilized for 48 h at -55°C (Christ Alpha 1–2 LD plus, Osterode am Harz, Germany) in order to obtain TPP-HP-β-CD or TPP-SBE-β-CD complex powders (Miecznik and Kaczmarek, 2007; Pant and Negi, 2018). The complexes were kept in tightly sealed glass vials at 4°C for further use.

Characterization of TPP-HP-β-CD and TPP-SBE-β-CD inclusion complexes

TPP-HP-β-CD or TPP-SBE-β-CD complexes were characterized by Fourier-Transform Infrared Spectroscopy (FT-IR), X-ray Powder Diffraction (XRD) and Differential Scanning Calorimetry (DSC) measurements, comparatively with physical mixtures.

FT-IR analysis studies were performed using a PerkinElmer Spectrum 100 FT-IR spectrometer (4000–550 cm^{-1}), comparatively with, TPP-HP-β-CD physical mixture, TPP-HP-β-CD complex, TPP-SBE-β-CD physical mixture and TPP-SBE-β-CD complex in the range of 550–4000 cm^{-1}.

The thermal profiles of the physical mixture and inclusion complexes were obtained with a Rigaku Ultima-IV X-ray diffractometer. The samples were scanned over a range of 5°–95° for XRD measurements.

DSC measurements were carried out with a Shimadzu DSC-DTA 60 at 10°C/min scan speed. The accurately weighed 2 mg of samples were sealed in an aluminium pan and equilibrated at 25°C. The samples were subjected to a heating run over the temperature range of 25-350°C.

The preparation of the CS nanoparticles using TPP, TPP-HP-β-CD and TPP-SBE-β-CD inclusion complexes

CS nanoparticles was carried out using the traditional ionic gelation method using LWCS and TPP (Calvo et al., 1997; Pant & Negi, 2018). In brief, the CS solution (0.125%) in 0.5% v/v acetic acid was prepared by overnight stirring at 500 rpm, room temperature. The CS solution was filtered through a 5 µm pore sized mixed cellulose esters membrane filter to remove coarse CS residues. The TPP solutions were prepared at a concentration range between 1 mg/mL-2.67 mg/mL to obtain the CS/TPP ratios as given in Table 1. Nanoparticles were formed with a dropwise addition of 2 mL of TPP solution in different con-

Table 1. CS-TPP nanoparticle formulations.

F. code	TPP amount (mg)	CS amount (mg)	CS/TPP ratio
F1	2.00	8	4
F2	2.29	8	3.5
F3	2.67	8	3
F4	3.20	8	2.5
F5	4.00	8	2
F6	5.33	8	1.5

centrations to 8 mL of prepared CS solution under continous stirring in a magnetic stirrer at 1000 rpm, room temperature for an hour. The preparation of nanoparticles is schematized in Figure 1.

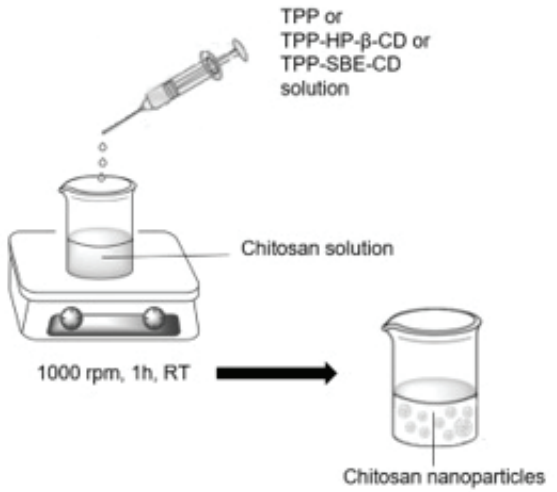

Figure 1. Preparation of CS nanoparticles.

To determine the effect of pH on nanoparticle formation, CS solutions (0.125%) were prepared in 0.5%, 0.25%, and 0.125% v/v acetic acid and used for CS-TPP nanoparticle formulation studies.

The concentrations of TPP were changed while the CS amount and the total volume were kept constant - 10 mL (Table 1). For the preparation of nanoparticles with TPP-HP-β-CD and TPP-SBE-β-CD inclusion complexes, the same procedure was followed and the equivalent TPP amounts of the complexes were calculated. According to the results of the CS-TPP nanoparticle preformulation studies, the optimum acetic acid ratio, CS/TPP ratio was chosen and used for preparing TPP-HP-β-CD or TPP-SBE-β-CD nanoparticles.

Particle size analysis and zeta potential measurements

The particle size (PS), poly dispersity index (PDI) and Zeta potential (ZP) of the CS nanoparticles were determined using a Malvern Zetasizer (Nano ZS90, Malvern instrument Ltd., UK). PS measurements were carried out in disposable polystyrene cuvettes while ZP measurements were carried out with folded capillary cells.

To confirm the effects of cyclodextrins on ionic gelation, the nanoparticles were prepared as mentioned previously and kept at room temperature. PS, PDI and ZP of the optimum CS-TPP and TPP-HP-β-CD nanoparticles were measured on the 0th, 1st, 3rd and 5th days following preparation.

Statistical analysis

All experiments were performed in triplicate and the results were presented as means ± standard deviation (SD). All results were analyzed by one-way ANOVA, and $p<0.05$ set as the minimal level of significance using GraphPad Prism 7.

RESULTS AND DISCUSSION

The TPP-HP-β-CD or TPP-SBE-β-CD complexes were prepared and characterized by FT-IR, XRD and DSC measurements comparatively with physical mixtures. Figure 2 represents the FTIR spectra of TPP-HP-β-CD, TPP-SBE-β-CD complexes and their physical mixtures. The spectrum of physical mixtures were different from the complexes as shown on the spectra. Changes at the absorption peaks at 1150 cm^{-1} and 863 cm^{-1} for both HP-β-CD/SBE-β-CD physical mixtures and complexes indicated the formation of the complexes.

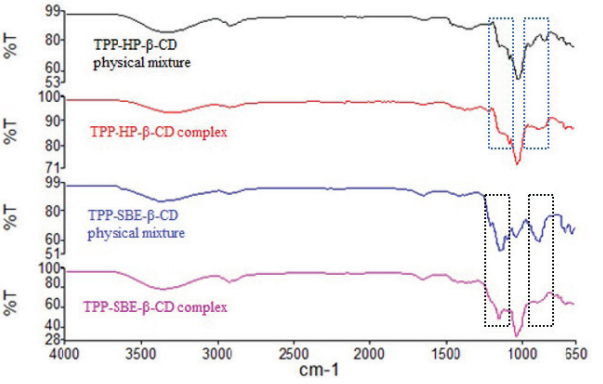

Figure 2. FT-IR spectra of TPP-HP-β-CD physical mixture (black line), TPP-HP-β-CD complex (red line), TPP-SBE-β-CD physical mixture (blue line) and TPP-SBE-β-CD complex (pink line).

As shown in Figure 3, for both TPP-HP-β-CD or TPP-SBE-β-CD complexes, the intensity of crystalline peaks in physical mixtures were significantly reduced. This result indicates a reduction of crystallinity and formation of an amorphous complex. However, neither TPP or HP-β-CD/ SBE-β-CD are water soluble, and the formed complexes are much more soluble as a result of the reduction in crystallinity (Kapor, 2008).

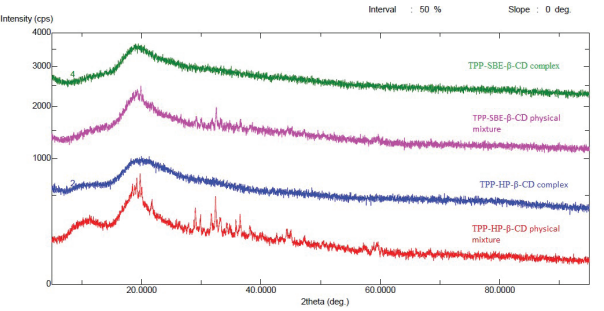

Figure 3. XRD diffractograms of TPP-HP-β-CD physical mixture (red line), TPP-HP-β-CD complex (blue line), TPP-SBE-β-CD physical mixture (pink line) and TPP-SBE-β-CD complex (green line).

The results of DSC analysis also supported the XRD results. According to the literature, TPP has a slight peak at around 200˚C. Although these slight peaks of TPP can be seen in Figure 4, at around 180˚C for physical mixtures, the peaks disappeared from the thermograms of the complexes, indicating the forma-

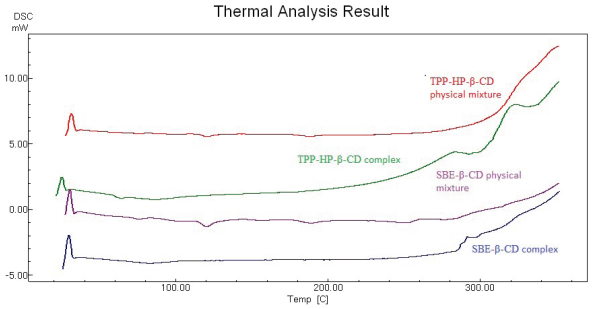

Figure 4. DSC thermograms of TPP-HP-β-CD physical mixture (red line), TPP-HP-β-CD complex (green line), TPP-SBE-β-CD physical mixture (purple line) and TPP-SBE-β-CD complex (blue line).

tion of the complexes (Nallamuthu et al., 2015). In addition, the peaks at around 280°C belonging to the cyclodextrins were much clearer for the complexes than the physical mixtures which also refers to the formation of the complexes.

The research of Calvo and his group, is one of the first studies that enlightens the conditions of CS nanoparticles prepared with TPP using the ionic gelation method (Calvo et al., 1997). Since then, there have been so many studies in literature on optimising CS nanoparticles. As a recent example, Sreekuomar and colleagues, investigated the effects of DA (degree of acetylation), DP (degree of polymerisation), NH_2/PO_4 molar ratio, and the concentration of the CS solution. It was reported that the optimum CS/TPP ratio, which this ratio denotes as the molar ratio of NH_2 groups of CS and PO_4 groups of TPP, was about 3 (Sreekumar et al., 2018).

Following the complex preparation, the CS nanoparticles were prepared with the traditional ionic gelation method using TPP, with varying CS/TPP ratios as shown in Table 1. It was considered to choose the optimum CS/TPP ratio and pH then use these parameters to prepare the CS-TPP-HP-β-CD and CS-TPP-SBE-β-CD nanoparticles.

Although the data is not shown, the CS ratios 0.5% and 0.25% were also tested but the particle sizes were found to be in micron sizes and the particle size increased proportionally with the CS concentration. Hence it was decided to use CS at 0.125%, to formulate the nanoparticles. It was clearly seen

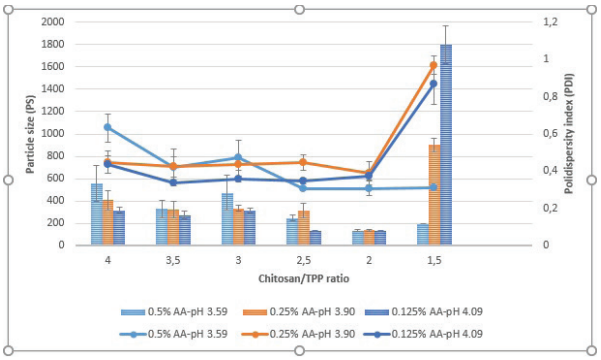

Figure 5. Effects of pH and CS/TPP ratio on PS and PDI (The bars represent changes in PS and the lines represent changes in PDI values).

that the CS/TPP ratio is the main factor affecting the nanoparticle size. The ratio 4 to 2, allows the formation of nanoparticles with varying sizes and PDIs values while the aggragates formed for the CS/TPP ratio 1.5 or lower at higher pHs, 3.90 and 4.09 (Figure 5).

On the other hand, the ZP values were found to be directly proportional to CS/TPP ratio, as the CS/TPP ratio decreases, the ZP values decreases (Figure 6). While the ZP value of the CS/TPP ratio 4 (0.5% AA) formulation was 35.9±1.42 mV, the CS/TPP ratio 1.5 (0.5% AA) formulation was found to be 12.4±0.305 mV. As the CS/TPP ratio decreased below 3, the changes in ZP values were found to be significant ($p<0.05$).

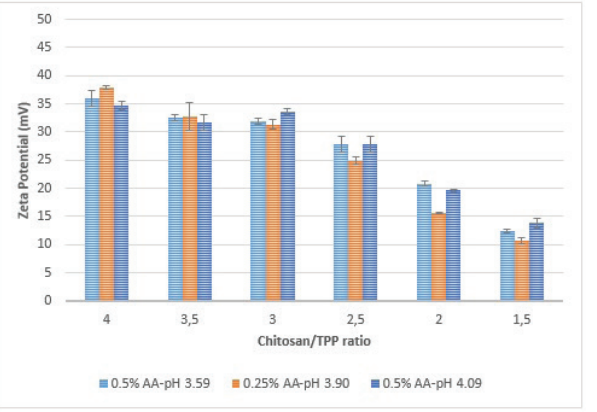

Figure 6. Relation of ZP values of formulations with varying CS/TPP ratios at different AA ratios.

According to the findings, the optimum conditions for the preparation of CS/TPP were determined as given in Table 2. Using these conditions the nanoparticles were obtained with 144 nm PS, 0.317 PDI, 21.2 mV ZP.

Table 2. Parameters for optimum formulation.

Parameters	Optimum conditions
CS concentration	0.125%
Asetic acid (AA) ratio	0.5%
pH	3.59
CS/TPP ratio	2
Stirring rate	1000 rpm

The nanoparticles were prepared with TPP-HP-β-CD and TPP-SBE-β-CD using the conditions given in Table 2. The CS/TPP-HP-β-CD nanoparticles were found to be preferable compared to the CS/TPP-SBE-β-CD nanoparticles due to their lower PS and PDI and higher ZP values (Table 3). CS-TPP-SBE-β-CD nanoparticles had also a tendency towards aggregation, such that aggregates with particle size over 2 µm could be easily seen a few hours after preparation. As both SBE-β-CD and TPP were anionic, complexing to CS caused a kind of neutralization and so aggregates could be

Table 3. Characteristics of final nanoparticles.

Nanoparticle type	PS (nm) ± SD	PDI ± SD	ZP (mV) ± SD
CS/TPP	144±22.9	0.317±0.0378	21.2±1.435
CS/TPP-HP-β-CD	178±84.1	0.310±0.0134	31.2±4.68
CS/TPP-SBE-β-CD	821.2±54.4	0.849±0.140	17.5±0.709

formed. There are studies in the literature about using SBE-β-CD instead of TPP as an anionic linker (Fulop et al., 2014; Mahmoud et al., 2011) suggesting its use as a linker itself rather than complexing with TPP.

Following the characterization of nanoparticles, the interday changes in PS, PDI and ZP of CS/TPP and CS/TPP-HP-β-CD nanoparticles were observed as a mini-stability study. As it is clearly seen in Figure 7, while the PS and PDI values of CS/TPP were increasing day by day ($p<0.05$), the values of CS/TPP-HP-β-CD did not significantly change ($p>0.1$). In addition, the ZP of CS/TPP nanoparticles dropped to 14.9 mV from 21.2 mV in 5 days, as an expected result because the initial ZP of TPP-HP-β-CD being significantly higher than the ZP of CS/TPP. The positive effect of TPP-HP-β-CD could be clearly seen to protect nanoparticle characteristics.

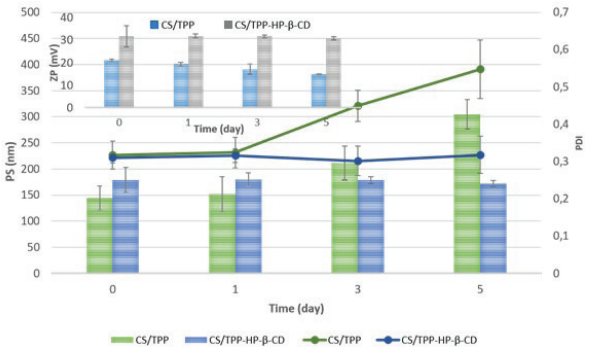

Figure 7. Interday changes in PS, PDI and ZP of CS/TPP and CS/TPP-HP-β-CD nanoparticles (The bars represent changes in PS and the lines represent changes in PDI values for PS-PDI time graph).

In a study by Pant and Negi in 2018, TPP-β-CD complexes were prepared for the first time in the literaure and used for the preparation of CS nanoparticles by ionic gelation method. The changes in the parameters PS, PDI and ZP were determined for 2 days and according to their results, the parameters changed significantly for the CS/TPP nanoparticles and β-CD enhanced interday stability (Pant and Negi, 2018). The results of this study also supported their findings, except their CS/TPP-β-CD nanoparticles were smaller than the CS/TPP nanoparticles. The MW of β-CD is lower than HP-β-CD, so this may be the reason for larger particle sizes of CS/TPP-HP-β-CD nanoparticles prepared in this study.

CONCLUSION

The ionic gelation method has been the most common and simple method for CS nanoparticle preparation since Calvo et al. first described this method for synthesis of CS nanoparticles using TPP. The method is basically related with the ionic interactions between CSs' amino groups and the phosphate groups of TPP. In the case of ionic interactions, undesirable complexations are also highly likely to occur. In this study, it was aimed to control those undesirable interactions using TPP-HP-β-CD complex instead of TPP and develop a nanocarrier for drug delivery studies. According to the results of the optimization study, optimum CS/TPP-HP-β-CD were obtained using a CS concentration 0.125% with 0.5% AA at a ratio of CS/TPP 2. CS/TPP-HP-β-CD nanoparticles were found to have a higher physical stability compared to CS/TPP nanoparticles as aimed. In conclusion, since cylodextrins are very well established molecules for enhancing solubility of hydrophobic drugs, CS/TPP-HP-β-CD nanoparticles with 178±84.1 PS and 31.2±4.68 mV would be a good candidate especially for hydrophobic drugs to improve their stability, solubility, encapsulation efficiency and in vivo bioavailability.

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

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Original Article

Evaluation of preservative efficacies of some unused cosmetic products

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ABSTRACT

Background and Aims: Cosmetics must be free of pathogenic microorganisms, and the total aerobic microbial count needs to be within acceptable limits.
Methods: In this study, preservative efficacies of ten commercially available cosmetic products were investigated against *Pseudomonas aeruginosa*, *Pseudomonas putida, Stenotrophomonas maltophilia* and *Burkholderia cepacia*, which were isolated from contaminated cosmetic products.
Results: According to our results, all products preservatives did not comply with the United States Pharmacopeia (USP) method recommended antimicrobial preservative activity criteria against at least one studied bacteria.
Conclusion: Consequently, according to our results, preservatives of unused cosmetic products can be ineffective against bacteria, especially bacteria isolated from cosmetics.

Keywords: Cosmetic, preservative efficacy, preservative

INTRODUCTION

According to the Turkish Cosmetic Regulation, a cosmetic product means any substance or mixture intended to be placed in contact with the external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view exclusively or mainly of cleaning them, perfuming them, changing their appearance, protecting them, keeping them in good condition or correcting body odours (Cosmetic Law, 2005). Cosmetics, like any product containing water and organic/inorganic compounds, are exposed to microbial contamination under appropriate conditions (Halla et al., 2018). Although sterility is not a requirement in cosmetics, they must be free of pathogenic microorganisms, and the total aerobic microbial count needs to be within acceptable limits (Mugoyela & Mwambete, 2010). Pathogenic organisms or high levels of saprophyte microorganisms in cosmetic products lead to spoilage, which is of great importance to the industry and also causes serious health risks for consumers (Campana, Scesa, Patrone, Vittoria, & Baffone, 2006).

Studies have shown that many bacteria and fungi can be found as contaminants in cosmetics. Among them, pathogenic Gram-negative bacteria *Pseudomonas sp., Stenotrophomonas maltophilia* and *Burkholderia cepacia* are the most common contaminants found in cosmetics, which are infectors of wounds and burns, and also cause pneumonia, especially in immunosuppressive patients. These bacteria are commonly isolated nosocomial pathogens, and *Pseudomonas sp.* are also responsible for a variety of infectious diseases affecting the eyes and surrounding tissues (corneal ulcer, bacterial keratitis) and may cause loss of sight (Brannan, 2006; Neza & Centini, 2016).

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Therefore, in order to prevent the microbial contamination, which is the biological and physicochemical deterioration of a cosmetic product, manufacturers need to use chemical preservatives with known antimicrobial properties (Sutton, 2006). Several preservatives are used to ensure the microbiological quality, consumer safety and organoleptic properties of cosmetic products (Orús, Gomez-Perez, Leranoz, & Berlanga, 2015). In our country, according to the Cosmetic Law (Law No: 5324) and Regulations/Appendixes, a list of allowed preservatives in cosmetic products with maximum concentrations in ready-for-use preparations are listed, and manufacturers have to comply with these (Cosmetic Law, 2005).

Commonly used preservatives in cosmetics are formaldehyde releasers, isothiazolinones, organic acids and alcohols. An ideal preservative should be effective in low concentrations against a wide variety of microorganisms, non-toxic and compatible with other ingredients (Geis, 2006). Due to alkyl esters of p-hydroxybenzoic acid, although parabens are excellent preservatives with antimicrobial activity, side effects on the endocrine and the reproductive systems have limited their use in cosmetics (Darbre et al., 2002; Kizhedath, Wilkinson, & Glassey, 2019).

In this study, we aimed to investigate the antimicrobial preservation efficacy of various commercially available cosmetic products against *Pseudomonas aeruginosa, Pseudomonas putida, S. maltophilia* and *B. cepacia* which are involved in recurrent contamination in cosmetic products containing preservatives.

MATERIAL AND METHODS

Cosmetic products

Ten commercially available cosmetic products (4 liquid soaps, 3 shampoos and 3 make-up removers) were collected and employed in the study. Samples were bought from markets and analyzed as soon as possible upon their arrival.

Preservatives of the cosmetic products

Three of the liquid soaps contained Dimethyl dimethylol hydantoin (DMDM hydantoin), methylisothiazolinone and methylchloroisothiazolinone and one liquid soap contained methylisothiazolinone and methylchloroisothiazolinone, sodium benzoate and potassium sorbate. Although all three shampoos contained sodium benzoate, one of them also contained phenoxyethanol, and additionally, one of them also contained potassium sorbate, DMDM hydantoin, methylisothiazolinone and sodium salicylate. Two make-up removers contained polyaminopropyl biguanide, and one contained phenoxyethanol.

Neutralizer

The neutralizer used in this study contained lecithin, Polysorbate 80, sodium thiosulfate pentahydrate, L-Histidine, proteose peptone, sodium chloride, $Na_2HPO_4.12\ H_2O$ and KH_2PO_4.

Validation of neutralizer

The validation method for neutralizing the antimicrobial properties of a product must meet two criteria, neutralizer efficacy (NE) and neutralizer toxicity (NT). One mL of the test sample (test group) or peptone (peptone group) was added to a tube containing 9 mL of neutralizing broth. A third tube, containing 10 mL sterile saline (viability control) and a fourth tube containing one ml test sample and 9 ml saline solution (dilution control) were also prepared. Each of these solutions was inoculated with $1x10^4$ colony forming units/ml (cfu) of the challenge organisms, and incubated for 10 minutes on the benchtop at ambient temperature. Recovery of all organisms was determined by the plate count on tryptic soy agar (TSA, Difco Laboratories), and the plates were incubated at 30-35°C for three days. NT was determined by comparing the recovery of the microorganisms in the peptone group and the viability group. NE was determined by comparing the number of the recovered microorganisms in the test group and peptone group, and also by dilution control and viability control. An effective and non-toxic neutralizer was defined by NE and NT ratios of > or=0.70 (USP, 2006).

Challenge test for preservative efficacy

The efficacy of antimicrobial preservation of cosmetic products was investigated as suggested by USP. *P. putida, S. maltophilia* and *B. cepacia* which were isolated from contaminated cosmetic products from moisturizing cream, toothpaste and face care cream, respectively, and a standard strain *P. aeruginosa* ATCC 9027, were used in this study (Birteksoz, Tuysuz, & Otuk,2013). Freshly grown bacteria were harvested in sterile tryptone sodium chloride, and prepared with $1x10^8$ cfu/ml inoculums. Each sample weighed 50 grams in aseptic state and was inoculated respectively with 0.5 ml of each inoculum suspensions. All inoculated samples were shaken and incubated at 25°C for 28 days. Aseptic samples were removed on days 0, 14 and 28 to the efficient and nontoxic neutralizing medium which is verified above. Numbers of viable microorganisms, in the inoculum suspension, were determined by the plate count by using Dey-Engley Neutralizing Agar (Difco Laboratories), and the plates were incubated at 37°C 24-48h. At the end of the incubation period, the number of colonies was recorded for each plate, and counts were expressed as cfu/g. The acceptance criteria were at least the second logarithmic reduction from initial count and no increase from the 14 days' count at 28 days (USP, 2006).

RESULTS

Validation of neutralizer

According to NE and NT ratios of > or=0.70, the neutralizer used in this study was found efficient and non-toxic to studied microorganisms. Validation results were shown at Table 1.

Table 1. NE and NT rations of the neutralizer

	NT rations	NE rations	
P. aeruginosa ATCC 9027	0.9	0.8	0.75
P. putida	0.92	0.88	0.77
S. maltophilia	0.74	0.9	0.78
B. cepacia	0.8	0.92	0.83

Antimicrobial efficacy test

The results were evaluated according to USP, with no less than 2 log reductions from the initial count in 14 days and no more from the 14 days' count in 28 days. Ten products were studied, and it was detected that none of the products preservative complied with the USP recommendations for antimicrobial preservative activity criteria against at least one studied bacterium. Preservatives of one liquid soap, two shampoos and all the make-up removers were found to be ineffective against *P. aeruginosa* and three liquid soaps, all the shampoos and one make-up remover were found ineffective against *P. putida*. It was detected that the preservatives of three shampoos against *B. cepacia* and the preservatives of two liquid soaps, one shampoo and two make-up removers were found ineffective against *S. maltophilia* (Figure 1a-d).

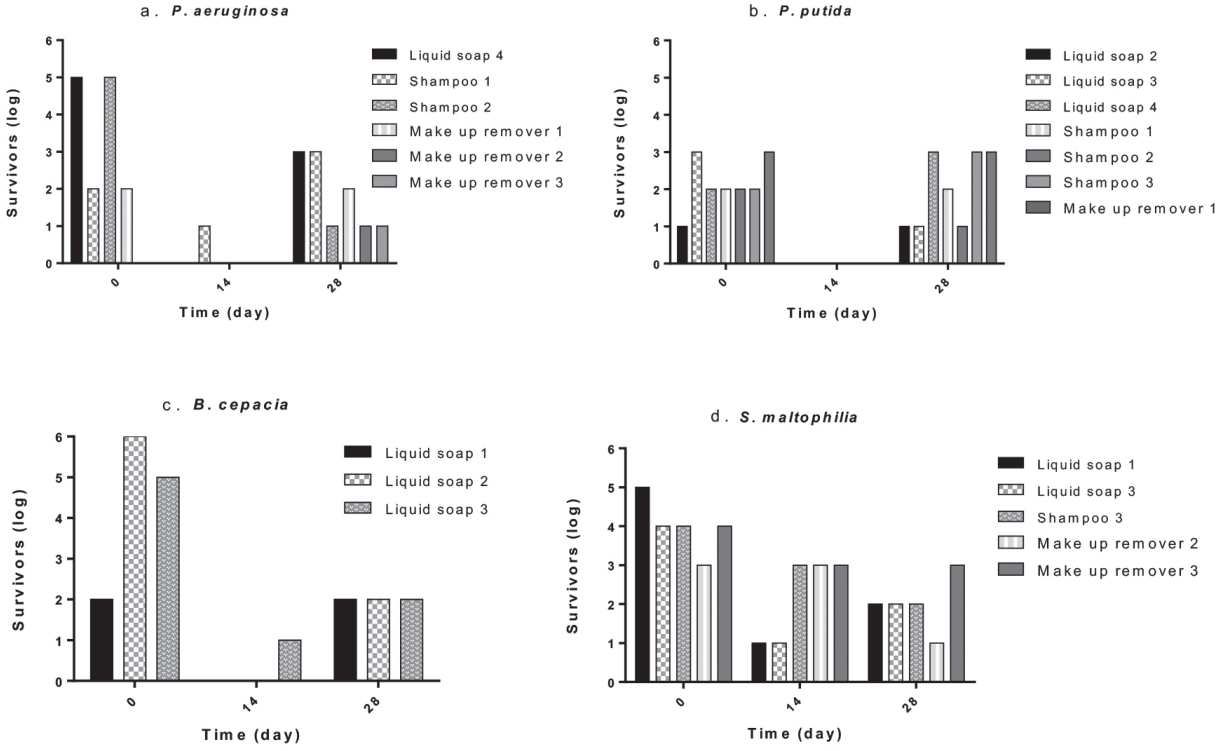

Figure 1. Microorganisms survival in cosmetic products. (a) *P. aeruginosa*, (b) *P. putida* (c) *B. cepacia*, (d) *S. maltophilia*.

DISCUSSION

Since consumer health is the primary concern, product quality can be improved and the risk of microbiological contamination can be prevented by using preservatives (Geis, 2006). In this study, we investigated the preservative efficacies of ten commercially available and unopened cosmetic products. According to our results, all the products' preservatives did not comply with the USP recommended antimicrobial preservative activity criteria against at least one of the studied bacteria.

The preservatives of a liquid soap, methylisothiazolinone and methylchloroisothiazolinone and sodium benzoate and potassium sorbate, preservatives of two shampoos, phenoxyethanol, sodium benzoate, potassium sorbate, DMDM hydantoin, methylisothiazolinone and sodium salicylate and the preservatives of make-up removers, polyaminopropyl biguanide and phenoxyethanol were found ineffective against *P. aeruginosa*. DMDM hydantoin, methylisothiazolinone, methylchloroisothiazolinone, sodium benzoate and potassium sorbate which were the preservatives of three liquid soaps, DMDM hydantoin, methylisothiazolinone, potassium sorbate, sodium salicylate, phenoxyethanol and sodium benzoate, preservatives of three shampoos and phenoxyethanol, preservative of a make-up remover were found ineffective against *P. putida*.

Pseudomonas sp. such as *P. aeruginosa* and *P. putida* are frequently found in contaminated cosmetics. In contaminated ophthalmic preparations, *P. aeruginosa* is also responsible for serious eye infections (corneal ulcer, bacterial keratitis), and even loss of vision (Birteksoz Tan et al.,2013; Brannan, 2006; Neza & Centini, 2016; Yossa et al., 2018). Furthermore, Hopfer et al. reported infections and one death due to a *P. aeruginosa* contaminated shampoo used by immunosuppressed patients (Geis, 2006).

An opportunistic pathogen, *B. cepacia,* is also a frequent contaminant in cosmetics, and it is one of the causes of product recalls (Alvarez-Lerma et al., 2008; Birteksoz Tan et al.; 2013, Jimenez, Smalls, Jimenez, & Smalls, 2000). Three shampoos' preservatives DMDM hydantoin, methylisothiazolinone and methylchloroisothiazolinone were found ineffective against *B. cepacia* in our study.

One of the important causes of nosocomial infections, *S. maltophilia*, can be found in used cosmetics such as shampoos or body lotions (Birteksoz Tan et al., 2013; Brannan & Dille, 1990).

In our study, the preservatives of two liquid soaps, DMDM hydantoin, methylisothiazolinone, methylchloroisothiazolinone, the preservatives of a shampoo, sodium benzoate and the preservatives of two make up removers, polyaminopropyl biguanide, were found ineffective against *S. maltophilia***.**

Phenoxyethanol is one of the most commonly used preservatives in personal care formulations, but it was found ineffective against both *P. aeruginosa* and *P. putida*. Similar to our results, Flores et al. (1997) found several microorganisms which were isolated from contaminated cosmetic products, resistant to phenoxyethanol.

Although among the formaldehyde releasers, DMDM hydantoin, and among the isothiazolinones, methylisothiazolinone and methylchloroisothiazolinone, are some of the most common and effective preservatives, they were found ineffective against at least one of the studied bacteria, in our study.

Because the development of resistance to preservatives is not a new phenomenon and it is a problem with serious economic and health consequences, new researches should focus on alternative or new preservatives (Chapman, 1998).

Consequently, according to our results, it has been showed that preservatives of unused cosmetic products can be ineffective against bacteria, especially bacteria isolated from cosmetics. Preservatives should be added to cosmetic products as determined by regulations, and in accordance with toxic dose limits, for consumer's health, and they should also be investigated for their effectiveness against the most isolated bacteria.

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Original Article

Understanding of Turkish pharmacists health literacy knowledge, attitudes, and behavior

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ABSTRACT

Background and Aims: Health literacy is a set of cognitive-social skills and motivation to access, understand, and utilize information to protect and improve the health of individuals. Pharmacists, as public health advisors, need to play an active and effective role in preventing the negative consequences of inadequate health literacy. In this study, we determined the knowledge, attitudes, behaviours, and educational needs related to pharmacists' health literacy.
Methods: This cross-sectional study was conducted in cities across Turkey. According to the '2016 Turkish Pharmacists Association Database', in 2015 and 2016, there were 25,453 and 24,928 private pharmacists, respectively. To calculate the sample size, we used a population of 24,928, a 50% expected prevalence (unknown frequency), a 5% deviation value, a 95% confidence interval, and a 1.0 design effect. The necessary sample size was determined to be 379. Using a random number table, we recruited 398 participants for this study.
Results: Our results revealed that the pharmacists who participated in this study stated high awareness of health literacy.
Conclusion: In healthcare facilities, pharmacists should play an active role in promoting and delivering health literacy.

Keywords: Pharmacist, health literacy, knowledge, attitude, behaviour

INTRODUCTION

According to the World Health Organization (WHO), health literacy is a set of cognitive-social skills which determine the motivation and ability of individuals to gain access to, understand, and utilize information to protect and improve their own health (Kickbusch & Nutbeam, 1998); and includes understanding complex readings, gaining analytical and decision-making skills, and using them in health-related situations (U.S. Department of Health and Human Services, 2010). WHO explains the close relationship between health literacy and general literacy as follows:

Health literacy is associated with general literacy and it is the will and capability of people in developing and deciding on issues related to health services throughout their lives, maintaining and improving their health, reaching health-related information sources to improve their quality of life, accurately perceiving and understanding health-related information and messages (Kickbusch, Pelikan, Apfel, & Tsouros, 2013).

Studies in the United States and within the European Union have shown that nearly 50% of the population in developed countries have poor health literacy, and this rate reached up to 70% in some studies performed in Turkey (U.S. Department of Health and Human Services 2010, Kickbush et al., 2013). A study conducted in eight European countries (Germany, Austria, Bulgaria, the Netherlands, Spain, Ireland, Poland, and Greece) that examined health literacy found that 12% of the participants had an insuf-

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ficient health literacy level and an additional 23% had problematic health literacy levels. Compared to their counterparts, health literacy levels are also lower in groups with low general education and income levels, in minority groups, in recently migrated groups, in groups with low general health levels, in patients with long-term health problems, and among the elderly (Kickbush et al., 2013).

Thus, health literacy, which has been on the agenda in Turkey for the last five years (Tanrıover, Yıldırım, Ready, Cakır & Akalın, 2014), is a critical issue that requires study to increase individuals' responsibility for their own health, and must be considered when developing health services. Key components of the Ministry of Health's strategic plan (2013-2017) were "to develop health literacy to increase the responsibility of individuals for their own health," "to protect individuals and society from health risks and to promote a healthy lifestyle," and "to prepare programs for health literacy educators and trainers to improve their capacity" (T.C. Sağlık Bakanlığı, 2012).

Regarding this aim to increase health literacy levels in society, there are many stakeholders, but the main ones are patients (society) and health professionals because the health services outputs pass between them. Additionally, pharmacists are the health professionals in close contact with the patients, so they are required to develop relevant knowledge, attitudes and behaviors – skills that can be learned either before and/or after graduation - to deal with the responsibility of improving health literacy levels in society. So, in order to contribute to graduate and postgraduate educational programs, to determine which intervention practices require analysis, and to contribute to the increase in health literacy levels, we believe it is of utmost importance to learn how pharmacists understand the concept of health literacy, to determine their level of skills related to this concept and the possible existent gaps in these skills.

Aim of the study

In this study, we aimed to gather and analyze data regarding Turkish pharmacists' understanding of health literacy related to the general population, to determine the level of their knowledge, attitudes, and behaviors concerning health literacy and the gaps that may exist in these skills.

METHODS

This study was characterized as a cross-sectional research. According to data from the 2016 Turkish Pharmacists' Association Database, there were 24,928 private pharmacists in 2016. To calculate the sample size, we used a population of 24,928, a 50% "positive approach to pharmacists' health literacy" expected prevalence (unknown frequency), a 5% deviation value, a 95% confidence interval, and a 1.0 design effect. The necessary sample size was determined to be 379. Using a random number table, we interviewed 398 participants for this study.

In Turkey, all private pharmacists are members of the union, so the Association has a list of e-mail addresses and telephone numbers, which was utilized for this study. Then, we sent informed consent forms to all participants by e-mail and collected the signed pdf version. The survey was conducted by telephone.

The questionnaire used to collect data was entitled "The study on the determination of knowledge, attitude, behavior, and educational needs of pharmacists about health literacy." It was developed by the researchers based on the literature (Okyay & Abacıgil, 2016), containing three sections:

1. A section with 20 sociodemographic questions;

2. A section containing 25 suggestions (Table 1) used to determine the knowledge, attitudes, and behaviors of pharmacists on health literacy, measured using a five-point Likert-type scale (strongly disagree=1, disagree=2, neutral=3, agree=4, and definitely agree=5). In total, 5 knowledge, 5 attitudes, and 15 behavior suggestions were used.

3. A section containing eight suggestions related to the difficulties that prevent healthy communication among patients with inadequate health literacy, and they were measured using a 5-point Likert-type scale (strongly ineffective=1, ineffective=2, neutral=3, effective=4, and strongly effective=5).

The data were analyzed using SPSS Statistics for Windows, Version 18.0 (SPSS Inc., Chicago, IL, USA). The normality of the distribution of variables was analyzed using visual (histogram and probability graphs) and analytic methods (Kolmogorov-Smirnov/Shapiro-Wilk tests). Descriptive statistics are expressed as means (±) standard deviations, frequencies, and percentages. Statistical methods included Student's t-test, analysis of variance, Kruskal–Wallis analysis of variance, chi-square test, Mann–Whitney U Test, and logistic regression. The five points from the Likert scale were collapsed into three points by combining strongly agree/agree and strongly disagree/disagree. Statistical significance was set at $p < .05$.

RESULTS

The mean age of the 398 pharmacists in the study were 43.4±8.7 years (median = 46 years; range= 26–67 years). Among the participants, 9% were aged 18–29 years, 22.6% were aged 30–39 years, 48.2% were aged 40–49 years, 19.1% were aged 50–64 years, and 1.1% were aged ≥ 65 years. Moreover, 58.8% were women and 41.2% were men. Concerning location, pharmacists from 17 different cities participated: Majority being from Ankara (34.7%), 10.1% from Istanbul, and 9% from Çanakkale.

Pharmacists' knowledge, attitude, and behavior scores concerning health literacy according to age and sex are shown in Table 2. Attitude scores were higher when participants were younger. There was a significant increase in behavior scores after the age of 40 years before stabilizing. There were significant differences between the age groups in all three components of health literacy. There were no significant sex differences; however, behavior health literacy scores were higher in female pharmacists as compared to male pharmacists ($p < .05$).

Table 1. Questions used to determine the knowledge, attitude, and behavior of pharmacists about health literacy.

Knowledge	• I know what low health literacy means for patients • I know the frequency of low health literacy • I know what groups are prone to low health literacy • I know the relationship between health literacy and health • I know how to treat patients with poor health literacy
Attitude	• Inadequate health literacy is an important public health problem • As a health worker, I have responsibilities arising from the levels of my patients' health literacy • Studies to improve health literacy should be conducted and programs should be developed • Studies aimed at improving health literacy will affect the quality of health services • Studies aimed at improving health literacy will affect the occupational satisfaction of health workers
Behavior (skill)	• I create an environment that respects the privacy of the individual during drug counseling • I make sure that the patient is seated in a suitable place if necessary • I address patients with their names • I know individuals with low health literacy • I know what information I have told my patients that can be understood by the patients • I speak slowly • I am careful not to use medical terms • I repeat the information that I provide • I want my patients to repeat or show me what I say or suggest • I highlight a maximum number of key points (1 to 3) during a conversation • When giving information to my patients, I show/draw with pictures/writing when necessary • I create patient-specific training material • I use improved training and information materials (brochures, booklets, etc.) • I highlight key points in the information materials that I use • I direct patients to health information sources that I think are appropriate

Table 2. Pharmacists' knowledge, attitude, and behavior scores concerning health literacy according to age and sex (N=398).

Age group and gender		Knowledge score Median (min-max)	Attitude score Median (min-max)	Behavior score Median (min-max)
Age group	18–29 years	19.0 (14.0–24.0)	24.0 (20.0–25.0)	64.0 (52.0–69.0)
	30–39 years	21.0 (18.0–25.0)	22.0 (17.0–25.0)	62.0 (46.0–72.0)
	40–49 years	21.0 (10.0–25.0)	23.0 (14.0–25.0)	65.5 (53.0–73.0)
	50–64 years	21.0 (19.0–23.0)	22.0 (20.0–25.0)	65.0 (47.0–72.0)
	≥65 years	20.0 (20.0–20.0)	21.0 (21.0–21.0)	65.0 (65.0–65.0)
		p=0.0001[a]	**p=0.016[a]**	**p=0.0001[a]**
Sex	Male	21.0 (14.0–25.0)	22.5 (17.0–25.0)	64.0 (46.0–73.0)
	Female	21.0 (10.0–25.0)	22.0 (14.0–25.0)	65.0 (53.0–73.0)
		p=0.063[b]	**p=0.063[b]**	**p=0.341[b]**

[a]Kruskal–Wallis analysis of variance; [b]Mann–Whitney U Test; N: Number of respondents

Overall, 17.6% of the pharmacists spent less than 10 years in the profession, 24.1% had worked 10–19 years, and 58.3% had worked more than 20 years. Almost 72.9% had worked in the same place for 5 years or more and 27.1% had worked there for less than 5 years. Only four had an assistant pharmacist. The number of daily patients was < 20 among 1.5% of the pharmacists, 20–29 among 2%, 30–39 among 8.1%, 40–49 among 37.2%, 50–59 among 25.6%, and ≥ 60 among 25.6% of the pharmacists. Pharmacists' knowledge, attitude, and behavior scores concerning health literacy according to years spent in the occupation, years spent at the current workplace, and number of patients seen daily are shown in Table 3. Significant differences were seen for all factors, except for participants' attitude scores and the number of patients seen daily.

The satisfaction levels of pharmacists were as follows: 1% were dissatisfied, 8.5% were partially satisfied, 1.5% were neutral, 49.2% were satisfied, and 39.8% were very satisfied.

Table 3. Pharmacists'knowledge, attitude, and behavior scores concerning health literacy according to years spent in the occupation, years spent at the current workplace, and number of patients seen daily (N=398).

Working time and number of patients		Knowledge score Median (min-max)	Attitude score Median (min-max)	Behavior Median (min-max)
Time spent in the occupation	<10 years	19.0 (14.0–25.0)	22.0 (17.0–25.0)	64.0 (50.0–71.0)
	10–19 years	21.0 (18.0–25.0)	22.0 (19.0–25.0)	65.0 (46.0–73.0)
	≥20 years	21.0 (10.0–25.0)	22.5 (14.0–25.0)	65.0 (47.0–73.0)
		p=0.0001[a]	**p=0.0001[a]**	**p=0.024[a]**
Time spent in the current workplace	≤5 years	20.5 (14.0–25.0)	21.0 (17.0–25.0)	64.0 (50.0–72.0)
	>5 years	21.0 (10.0–25.0)	23.0 (14.0–25.0)	65.0 (46.0–73.0)
		p=0.004[b]	**p=0.004[b]**	**p=0.001[b]**
Number of patients seen daily	<40	21.0 (14.0–25.0)	64.0 (55.0–71.0)	21.0 (17.0–25.0)
	≥40	21.0 (10.0–25.0)	65.0 (46.0–73.0)	22.0 (14.0–25.0)
		p=0.037[a]	**p=0.245[a]**	**p=0.005[a]**

[a]Kruskal–Wallis analysis of variance; [b]Mann–Whitney U Test; N: Nnumber of respondents

Table 4. Pharmacists' knowledge, attitude, and behavior scores concerning health literacy according to in-service training and occupational satisfaction (N=398).

Training before or after graduation and occupational satisfaction		Knowledge score Median (min-max)	Attitude score Median (min-max)	Behavior score Median (min-max)
Receiving communication skills training before or after graduation	Yes	21.0 (17.0–25.0)	24.0 (18.0–25.0)	62.0 (50.0–73.0)
		21.0 (10.0–25.0)	22.0 (14.0–25.0)	65.0 (46.0–72.0)
		p=0.0001[b]	**p=0.0001[b]**	**p=0.016[b]**
Health literacy training before or after graduation	Yes	22.6 (20.0–25.0)	22.0 (19.0–25.0)	62.0 (55.0–70.0)
	No	21.0 (10.0–25.0)	22.0 (14.0–25.0)	65.0 (46.0–73.0)
		p=0.0001[b]	**p=0.0001[b]**	**p=0.063[b]**
Occupational satisfaction	Dissatisfied	20.0 (10.0–25.0)	62.0 (53.0–72.0)	21.0 (14.0–25.0)
	Neutral	21.0 (20.0–25.0)	65.0 (61.0–72.0)	23.0 (19.0–25.0)
	Satisfied	21.0 (17.0–25.0)	65.0 (46.0–73.0)	22.0 (17.0–25.0)
		p=0.008[a]	**p=0.102[a]**	**p=0.041[a]**

[a]Kruskal–Wallis analysis of variance; [b]Mann–Whitney U Test; N: Number of respondents

Pharmacists' knowledge, attitude, and behavior scores concerning health literacy according to in-service training and occupational satisfaction are shown in Table 4. Significant differences were seen for all factors, except for participants' behavior scores, attitude scores, health literacy training before or after graduation, and occupational satisfaction.

Of the pharmacists who participated in this study, 87.4% stated that they had not received education related to health literacy, 78.4% had received communication skills training before or after graduation, 86.9% thought that they knew the rights of patients while offering healthcare, and 84.9% agreed that they knew what low health literacy meant for patients. The pharmacists' opinions regarding attitudes about health literacy and behaviors related to health literacy are shown in Tables 5 and 6, respectively.

Pharmacists' opinions concerning the difficulties that prevent healthy communication among patients with inadequate health literacy are shown in Table 7.

When pharmacists were asked what subjects should be included in training, the critical issues were media literacy, special education programs for chronic diseases, educational programs for preventive health services, programs for multi-drug use, respect for the pharmacist profession, informing the public about practices resulting from health practice communications, and informing pharmacists about local terms for certain medical conditions.

Table 5. Pharmacists' opinions regarding attitudes about health literacy (N=398).

Opinions	n (%)
Inadequate health literacy is a key public health problem	
Disagree	4 (1.0)
Neutral	32 (8.0)
Agree	362 (91.0)
As a health worker, I have responsibilities arising from my patients' health literacy	
Disagree	8 (2.0)
Neutral	56 (14.1)
Agree	334 (83.9)
Studies to improve health literacy should be conducted and programs should be developed	
Disagree	4 (1.0)
Neutral	20 (5.0)
Agree	374 (94.0)
Studies to improve health literacy will affect the quality of health services	
Disagree	-
Neutral	12 (3.0)
Agree	386 (97.0)
Studies aimed at improving health literacy will affect the occupational satisfaction of health workers	
Disagree	-
Neutral	32 (8.0)
Agree	366 (92.0)

N: Number of participants, n: Number of respondents, %: Percentage of column

DISCUSSION

When analyzing these study findings, we noted that most pharmacists were satisfied with their profession, and most had never received any education related to health literacy; however, most had received communication skills training before or after graduation.

A significant proportion of the pharmacists involved in this study had a high awareness of health literacy. More than half stated that they understood what low health literacy meant for patients, they knew the frequency of low health literacy, knew what groups were prone to low health literacy, understood the relationship between low health literacy and health, and knew how to treat patients with low health literacy. In addition, most thought that inadequate health literacy was a key public health problem; that, as healthcare workers, they had responsibilities arising from their patients' health literacy; thought that studies should be performed to improve health literacy; and thought that these studies would affect healthcare quality and professional satisfaction. The 49.2% pharmacists were satisfied with their jobs.

Almost all pharmacists agreed that "inadequate health literacy is a major public health problem," that "studies to improve health literacy should be conducted and programs should be developed," that "studies to improve health literacy would affect the quality of health services," and that "studies aimed at improving health literacy will affect the occupational satisfaction of health workers." In summary, we revealed an association between increased health literacy, health service satisfaction, and occupational satisfaction.

In practical terms, we found data that would corroborate the idea that health care could be enhanced by improving the health literacy level of both pharmacists and patients, which could lead to an improvement in the rationality and cost-effectiveness of drug use. Thus, we can infer that, if the level of health literacy were adequate in Turkey, this could positively affect the health care system costs. Further, health professionals with good health literacy could improve their patients' health literacy level and thereby help the latter to obtain more efficient and effective health care. With that in mind, we believe that efforts to improve health literacy need to be multifaceted. The Ministry of Health, media outlets, health professional organizations, universities, and health vocational schools should participate actively and effectively in these studies.

Also, when measuring health literacy, it is essential to use a scale that considers respondents' sociocultural and economic characteristics. In Turkey, patients can easily reach pharmacies, and pharmacists act as health consultants in many health problems. Therefore, the knowledge, attitude, and behavior of pharmacists concerning health literacy are critical in the dis-

Table 6. Pharmacists' opinions regarding behaviors related to health literacy (N=398).

Opinions	n (%)
During drug counseling, I create an environment that respects the privacy of the individual	
Disagree	-
Neutral	8 (2.0)
Agree	390 (98.0)
I know individuals with low health literacy	
Disagree	64 (16.1)
Neutral	60 (15.1)
Agree	274 (68.8)
I am careful not to use medical terms	
Disagree	4 (1.0)
Neutral	36 (9.0)
Agree	358 (90.0)
I repeat the information that I provide	
Disagree	-
Neutral	16 (4.0)
Agree	382 (96.0)
I want patients to repeat or show me what I say or suggest	
Disagree	12 (3.0)
Neutral	36 (9.0)
Agree	350 (88.0)
I show or draw with pictures or writing when I need to give information to patients	
Disagree	20 (5.0)
Neutral	28 (7.0)
Agree	358 (88.0)
I use developed training and information materials (brochures, booklets, etc.)	
Disagree	40 (10.0)
Neutral	40 (10.1)
Agree	318 (79.9)
I direct patients to health information sources that I think are appropriate	
Disagree	32 (8.0)
Neutral	40 (10.1)
Agree	326 (81.9)

N: Number of participants, n: Number of respondents, %: Percentage of column

Table 7. Pharmacists' opinions concerning the difficulties that prevent healthy communication among patients with inadequate health literacy (N=398).

Opinions	n (%)
Limited time per patient (N=398)	
Partially/slightly effective	12 (3.0)
Neutral	24 (6.0)
Effective	362 (91.0)
Health-related information is complicated (N=398)	
Partially/slightly effective	40 (10.1)
Neutral	16 (4.0)
Effective	342 (85.9)
Lots of information to be transferred (N=398)	
Partially/slightly effective	36 (9.0)
Neutral	28 (7.0)
Effective	334 (84.0)
Low knowledge or general education levels of patients (N=398)	
Partially/slightly effective	8 (2.0)
Neutral	16 (4.0)
Effective	374 (94.0)
Lack of prepared educational materials (N=398)	
Partially/slightly effective	8 (2.0)
Neutral	72 (18.1)
Effective	318 (79.9)
Insufficient efforts to improve health literacy (N=398)	
Partially/slightly effective	18 (4.5)
Neutral	28 (7.0)
Effective	352 (88.5)
Lack of available and appropriate public health information resources (N=398)	
Partially/slightly effective	16 (4.0)
Neutral	10 (5.0)
Effective	362 (91.0)
Health information in the media is misleading to patients (N=398)	
Partially/slightly effective	-
Neutral	16 (4.0)
Effective	382 (96.0)

N: Number of participants, n: Number of respondents, %: Percentage of column

semination of health services. To raise public health literacy to an adequate level, priority should be given to health professionals' ability to educate patients. Special informative materials should be prepared, training programs for health professionals should be developed, and these should be conducted on a platform common to all stakeholders.

The high frequency of agreement to the suggestions "showing information to patients with pictures or writing when necessary," "creating patient-specific written education materials," and "using improved information materials" also demonstrated the necessity to use and develop visual materials for postgraduate education to improve health literacy.

CONCLUSION

The main outcome of this study was to gather and analyze data to assess Turkish pharmacists' awareness in the health literacy topic related to the general population; to determine the level of their skills related to this concept; and to find possible gaps in the pharmacists' skills regarding health literacy that would require further training. In total, 398 Turkish pharmacists participated in this study, and we included male and female pharmacists from diverse age groups, and with a wide range of work experience.

Throughout this study, we found that communicating with patients and using visual materials increases the effectiveness of the message when communicating with patients regarding health literacy, and pharmacists indicated that these materials would be useful when presenting their opinions.

This study configures the first attempt to study this topic in Turkey regarding pharmacists, and we recognize this as its main strength. Now, in terms of limitations, we find that the opinions of the pharmacists were based on our suggestions, and there is a need for future studies to be conducted using scales and observers that may measure Turkish pharmacists' approach to health literacy. And also as the survey was conducted by telephone, it was unable to get information regarding behaviors. The majority of pharmacists that participated in this study were from the Ankara province though we planned to get information throughout Turkey.

Ethics Committee Approval: Our study protocol was approved by the Gazi University's Institutional Review Board on 11.04.2017, and all participants provided informed consent in the format required by the relevant board.

Informed Consent: Written consent was obtained from the participants.

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